(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: MEMBRANE MOLECULE INDICATOR COMPOSITIONS AND METHODS

(57) Abstract: The invention provides membrane molecule indicators, including polypeptides, encoding nucleic acid molecules and cells containing such polypeptides and nucleic acid molecules. The invention membrane molecule indicators are characterized in that fluorescence resonance energy transfer (FRET) between a donor fluorescent domain and an acceptor fluorescent domain indicates a property of the membrane molecule. Also provided are methods of using the invention membrane molecule indicators to determine a property of a membrane molecule, and to identify compounds that modulates a property of a membrane molecule.

WO 02/44720 A2

Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

MEMBRANE MOLECULE INDICATOR COMPOSITIONS AND METHODS

BACKGROUND OF THE INVENTION

The invention relates generally to the field
5   of signal transduction and, more specifically, to
compositions and methods for indicating properties of
membrane molecules using fluorescence resonance energy
transfer (FRET).

The transduction of signals from the outside
10   to the inside of a cell underlies most cellular
processes, including proliferation, differentiation,
apoptosis, motility and invasion. Therefore, there is
considerable interest in developing improved methods of
monitoring signal transduction in response to normal
15   and abnormal stimuli. Methods of monitoring signal
transduction have numerous applications, such as in
identifying or improving modulators of signal
transduction pathways, which are candidate therapeutic
drugs or therapeutic targets, and in detecting
20   pathological alterations in cells.

Some of the earliest and most sensitive
signals transduced in response to stimuli involve
changes in properties of membrane molecules, including
membrane lipids and polypeptides, such as changes in
25   location, abundance, conformation or post-translational
modification state. Accordingly, there exists a need
to develop compositions and methods suitable for
indicating changes in properties of membrane molecule.

An early response to agonist stimulation of
30   many tyrosine kinase and G-protein coupled receptors is
the activation of the enzyme phospholipase C, which

cleaves the lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to generate second messengers that increase cytosolic free Ca^{2+} concentration. Although Ca^{2+} indicators and methods have been described that allow

5 monitoring of Ca^{2+} concentration in single living cells with high spatial and temporal resolution, Ca^{2+} fluxes, being more distal to receptor activation, may not as faithfully report receptor activation levels as changes in PIP2 levels.

10   In a recently developed method for detecting PIP2 dynamics in living cells, a pleckstrin homology (PH) domain tagged with a green fluorescent protein (GFP) has been used. Detection of PIP2 hydrolysis was by *in vivo* visualization, such as by confocal imaging

15 and post acquisition image analysis, of translocation of the fluorescence from the membrane to the cytosol. However, this method suffers from several disadvantages. First, it is hard to obtain quantitative data using confocal microscopy, since even

20 minor focal drift and changes in cell morphology that often occur after stimulation render quantitative measurements unreliable. Second, it is difficult to visualize translocation in very flat cells or in cellular subregions. Third, at fast imaging rates,

25 confocal imaging requires high excitation intensities that can cause severe cell damage in minutes. Fourth, the imaging approach is not easily extended to cell populations. Therefore, there exists a need to develop improved methods for detecting PIP2 dynamics in cells,

30 and particulary methods amenable to high-throughput screening.

The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a membrane molecule indicator, the indicator containing:

(a) at least one membrane molecule indicator domain;

(b) a donor fluorescent domain; and

(c) an acceptor fluorescent domain;

wherein fluorescence resonance energy transfer (FRET) between the donor domain and the acceptor domain is indicative of a property of the membrane molecule.

Also provided is a nucleic acid molecule which encodes a membrane molecule indicator, or a nucleic acid kit, the nucleic acid molecule components of which encode a membrane molecule indicator, the indicator containing:

(a) at least one membrane molecule indicator domain;

(b) a donor fluorescent domain; and

(c) an acceptor fluorescent domain;

wherein FRET between the donor domain and the acceptor domain is indicative of a property of the membrane molecule.

The invention also provides a method of determining a property of a membrane molecule in a cell. The method includes the steps of:

(a) providing a cell containing a membrane molecule indicator; and

(b) determining FRET between the donor fluorescent domain and the acceptor fluorescent domain,

4

wherein FRET between the donor domain and the acceptor domain is indicative of a property of the membrane molecule.

Further provided is a method of identifying a compound that modulates a property of a membrane molecule. The method includes the steps of:

(a) contacting a cell containing a membrane molecule indicator with one or more test compounds, wherein the cell further comprises the membrane molecule; and

(b) determining FRET between the donor fluorescent domain and the acceptor fluorescent domain following the contacting,

wherein increased or decreased FRET following the contacting indicates that the test compound is a compound that modulates a property of the membrane molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows four exemplary membrane molecule indicator compositions. Solid bar: membrane anchoring domain. Hatched and open boxes: fluorescent donor domain or fluorescent acceptor domain. Thick semi-circle: MMID. Thin semi-circle: linker. Solid circle: membrane molecule. Solid triangle: represents an altered property of membrane molecule. (A-D): FRET is high due to association between membrane molecule indicator domain (MMID) and membrane molecule at the membrane. (E-H): FRET is low due to dissociation between MMID and membrane molecule, as a result of an altered property of membrane molecule. (I-L): FRET is low due to altered localization of membrane molecule.

Figure 2 shows fluorescence resonance
detection of PH domain translocation.   (A) Schematic
representation of FRET occurring between CFP-PH and
YFP-PH bound to the membrane.   Upon hydrolysis of
5   PI(4,5)P2, PH domains translocate to the cytosol and
FRET ceases.   (B) Emission signals of CFP and YFP,
collected at 475 and 530nm respectively, and the ratio
of 530/475, recorded from a single N1E-115 cell
stimulated with bradykinin (BK, 1 µM). Signals were
10   low-pass filtered at 2 Hz and sampled at 3 Hz. Scale
bar for ratio signal shows percent deviation from
baseline. (C) Confocal detection of GFP-PH
translocation, depicted on the same scale.   Images were
collected once per 10 seconds, and the ratio of
15   fluorescence intensities in membrane and cytosol
(PM/Cyt) was deduced for each image by post-acquisition
automated image analysis.

Figure 3 shows characterization of
fluorescence emission.   Cells expressing constructs as
20   indicated were stimulated with 1 µM bradykinin and
fluorescence emission was detected at the indicated
wavelength.

Figure 4 shows Fluorescence Recovery After
Photobleaching (FRAP) to reveal dynamic movements of
25   GFP-PH between cytosol and membrane.   Spots (approx.
1.3 µm full-width half maximum) were completely
bleached in the basal membrane (or in the cytosol for
B) with a 30 m-s pulse of 488 nm laser light, and
recovery was monitored in line-scan mode in a confocal
30   microscope. (A) FRAP of membrane-delimited YFP-CAAX;
(B) cytosolic PLCδ1PH(R40L)-GFP mutant that cannot bind
PI(4,5)P2; (C) PLCδ1PH-GFP in a resting cell; (D)
PLCδ1PH-GFP in a cell that has agonist-induced partial
translocation of fluorescence.   Insets show confocal

images for the distribution of these constructs, taken from distinct cells.

Figure 5 shows PLC activation in single cells, neurites or in cell populations recorded by
5   FRET. (A) A single N1E-115 cell was stimulated repeatedly with neurokinin A (NKA) as indicated by the lines (dashes, 10 s pulses of 100 µM NKA from a puffer pipette; solid line, addition of 1 µM final concentration to the culture dish). The response shows
10 repeated PLC activation and partial desensitization. PLC activation induced by subsequently added bradykinin (BK, 1 µM) was not desensitized by NKA pretreatment. For calibration, maximal translocation was induced by adding 5 µM ionomycin + 2 mM additional Ca^{2+}. (B) PLC
15 activation in a single neurite of a neuroblastoma cell, differentiated by culturing in serum-free medium for 48 hours. Area of measurement (2.5x9 µm) is indicated in the micrograph. Excitation bandwidth was increased to 20 nm. (C) FRET recording from a cluster of about 15
20 transfected cells demonstrates improved signal-to-noise ratio and averaged kinetics (note the same scale for B and C).

Figure 6 shows that the PH domain of PLCδ1 reports changes in PI(4,5)P2 rather than in IP3 in
25 N1E-115 cells. (A) Cells expressing GFP-PH were loaded with both Fura-Red (20 FM) and caged IP3 (100 PM) by in-situ high frequency electroporation. Shown is the response of a single cell, assayed simultaneously for GFP translocation and Ca^{2+} mobilization induced by
30 flash photolysis of caged IP3. Arrows indicate photolysis of 1 µM, 10 µM and 90 µM. For comparison, bradykinin (1 µM) was added afterwards. Representative trace from 16 similar experiments. (B) FRET response to bradykinin detected in a single cell, pretreated with 5

7

μM of phenyl arsine oxide for 10 minutes. (C-D), time
course of Ins(1,4,5)P3 and Ins(1,3,4)P3 formation in
adrenal glomerulosa cells prelabeled with [^{3}H]inositol,
after stimulation with angiotensin II (Ang, 1 μM) in
5   the presence of 2 mM Sr^{2+} or Ca^{2+}. (E) Angiotensin
II-induced translocation as quantitated by analysis of
serial confocal images of glomerulosa cells in the
presence of Sr^{2+} or Ca^{2+}. Data points represent means ±
S.E.M., n=5. (F) Bradykinin-induced translocation, with
10   and without Sr^{2+}, as detected by FRET in N1E-115 cells.

Figure 7 shows heterogeneity of PLC
activation responses to different GPCR agonists.
Single N1E-115 cells expressing CFP-PH and YFP-PH were
stimulated with 1 μM bradykinin (BK), 1 μM neurokinin A
15   (NKA), 50 μM thrombin-receptor activating peptide
(TRP), 1 μM lysophosphatidate (LPA) or 10 μM histamine
(HIS). PLC activation as assayed by FRET, and
intracellular Ca^{2+} recordings for these agonists
detected ratiometrically using Yellow Cameleon 2.1 in
20   separate experiments, are shown. Changes in
fluorescence ratio are expressed as percent of resting
values. Shown are representative examples of
experiments performed at least 10 times.

Figure 8 shows that PLC inactivation kinetics
25   mirror receptor inactivation. (A) FRET recording from
a single N1E-115 cell stimulated with neurokinin A
(NKA) and with 1 mM aluminum fluoride (AlF^{4-}). (B)
Confocal micrographs of cells, taken 56 hours after
transfection with PLCδ1PH-GFP (5 μg DNA/well) together
30   with different amounts of constitutively active Gαq
subunit (Gq*, 0.8 μg/well, and Gq* 1:10, 0.08 μg/well)
or with constitutively active Gα12 at 0.8 μg/well
(G12*). (C) PLC activation detected by FRET in single
neuroblastoma cells (left panel), expressing wild-type

8

NKA receptors, stimulated with 10 second pulse from a
puffer pipette with 100 µg NKA; and cells stimulated by
prolonged addition of NKA (1 µM) to the medium,
expressing either wild-type receptors (middle panel) or
5   a mutant truncated at its C-terminus (right panel).
Recordings are all to the same scale. (D) Kinetics of
PLC activation by NKA in a N1E-115 cell transfected
with the C terminally truncated NK2 receptors on an
extended time scale.

10   Figure 9 shows an exemplary membrane molecule
indicator. Oval: membrane molecule. Trapezoid: MMID.
The donor and acceptor fluorescent domains are
indicated. Top: FRET is high due to association
between MMID and the membrane molecule at the membrane
15   and proximity of the donor and acceptor. Bottom: FRET
is low due to relocalization of membrane molecule and
resulting separation of the donor and acceptor.

         Figure 10 shows an exemplary membrane
molecule indicator. Oval: membrane molecule.
20   Trapezoid: MMID. The donor and acceptor fluorescent
domains are indicated. Top: FRET is low due to
association between MMID and the membrane molecule at
the membrane and separation of the donor and acceptor.
Bottom: FRET is high due to relocalization of membrane
25   molecule and resulting proximity of the donor and
acceptor.

              DETAILED DESCRIPTION OF THE INVENTION

         The invention provides membrane molecule
indicator compositions, including polypeptides,
30   encoding nucleic acid molecules, and cells, as well as
related methods for determining properties of a

membrane molecule and for identifying. modulatory
compounds.

        The membrane molecule indicator compositions
of the invention are characterized by a membrane
5   molecule indicator domain, a donor fluorescent domain
and an acceptor fluorescent domain. The donor
fluorescent domain and acceptor fluorescent domain
exhibit a characteristic fluorescence resonance energy
transfer (FRET) when the membrane molecule indicator
10   domain is associated with a membrane molecule at a
membrane. This characteristic FRET observed when the
.membrane molecule indicator domain and membrane
molecule are associated at the membrane differs from
FRET observed when the membrane molecule indicator
15   domain dissociates from the membrane molecule, or when
the membrane molecule is no longer localized to the
membrane. Therefore, FRET between the donor and
acceptor fluorescent domains serves as an indicator of
association at the membrane between the membrane
20   molecule indicator domain and the membrane molecule,
and thus serves as an indicator of a property of the
membrane molecule.

        In one embodiment, FRET is high when the
membrane molecule indicator domain and membrane
25   molecule are associated at the plasma membrane (e.g.
Figure 1A-D and Figure 9, top), and low when the
membrane molecule indicator domain dissociates from the
membrane molecule (e.g. Figure 1E-H), or when the
membrane molecule relocalizes (e.g. Figure 1I-L and
30   Figure 9, bottom).

        In another embodiment, FRET is low when the
membrane molecule indicator domain and membrane
molecule are associated at the plasma membrane (e.g.

Figure 10, top), and high when the membrane molecule
indicator domain dissociates from the membrane
molecule, or when the membrane molecule relocalizes
(e.g. Figure 10, bottom).

5   Properties of a membrane molecule that can
affect its ability to associate at the membrane with an
indicator domain include, for example, its
localization, abundance, conformation and post-
translational modifications. These properties of
10   membrane molecules are of considerable interest, as
they often reflect changes that occur as a result of
activation or inactivation of cellular signaling
pathways that regulate fundamental cellular processes,
including growth, differentiation, apoptosis, motility
15   and invasion. Therefore, the invention compositions
and methods can be used to identify and determine the
function of modulators of cellular signaling pathways,
and thus have important therapeutic, diagnostic and
research applications.

20   In one embodiment, the membrane molecule
indicator compositions of the invention contain (or
encode) a single polypeptide that contains a membrane
molecule indicator domain, a membrane anchor, a donor
fluorescent domain and an acceptor fluorescent domain
25   (shown schematically in Figure 1A).

        In an alternative embodiment, the membrane
molecule indicator compositions of the invention
contain (or encode) two polypeptides, one containing a
membrane molecule indicator domain, the other
30   containing a membrane anchor domain, one of which
further contains a donor fluorescent domain, the other
of which further contains an acceptor fluorescent
domain (shown schematically in Figure 1B).

In another embodiment, the membrane molecule
indicator compositions of the invention contain (or
encode) a single polypeptide that contains two membrane
molecule indicator domains, a donor fluorescent domain

5   and an acceptor fluorescent domain (shown schematically
in Figure 1C).

In yet another embodiment, the membrane
molecule indicator compositions of the invention
contain (or encode) two polypeptides, each containing a

10   membrane molecule indicator domain, one of which
contains a donor fluorescent domain and the other of
which contains an acceptor fluorescent domain (shown
schematically in Figure 1D).

In a further embodiment, the membrane

15   molecule indicator compositions of the invention
contain (or encode) one polypeptide, containing a
central membrane molecule indicator domain, with a
donor fluorescent domain and an acceptor fluorescent
domain at the termini (shown schematically in Figures 9

20   and 10).

It will be appreciated by the skilled person
that the membrane molecule indicators shown in Figures
1, 9 and 10 can be modified in a variety of ways, so
long as the donor and fluorescent domains are operably

25   positioned so as to exhibit a characteristic FRET when
the membrane molecule indicator domain and membrane
molecule are associated at the membrane, which differs
from FRET observed when the membrane molecule indicator
domain dissociates from the membrane molecule, or when

30   the membrane molecule is no longer localized to the
membrane.

12

For example, the relative locations of the donor fluorescent domain and acceptor fluorescent domain with respect to a membrane anchoring domain can be reversed in the compositions shown in Figures 1A and
5   B. The membrane molecule indicator compositions can also contain additional peptide or non-peptide domains, such as linker sequences between the donor fluorescent domain and acceptor fluorescent domain, or between a fluorescent domain and either the MMID or the membrane
10   anchor. Likewise, either the donor or acceptor fluorescent domains shown in Figures 9 and 10 can optionally contain membrane anchor domains.

When two MMIDs are present, the MMIDs can each associate with the same type of membrane molecule.
15   In such applications, the MMIDs can be identical, or different, so long as they associate with the same type of membrane molecule. For other applications, it may be preferable that the MMIDs associate with different types of membrane molecules, which are commonly or
20   differentially regulated. Thus, such the membrane molecule indicator compositions can simultaneously, or alternatively, report the properties of two different membrane molecules.

As used herein, the term "membrane molecule"
25   refers to a molecule that transiently, or permanently, resides at, partially or completely within, or across, a lipid bilayer of a cell. A membrane molecule can thus be an integral membrane molecule, such as a lipid bilayer component or an integral membrane protein.
30   Alternatively, a membrane molecule can be a peripheral membrane molecule that directly associates with the lipid bilayer, or indirectly associates with the lipid bilayer by virtue of interaction with an integral membrane molecule.

A membrane molecule useful in the methods of
the invention is a molecule that as a direct or
indirect response to a normal or pathological stimulus,
exhibits a change in a property that results in an
5   increased or decreased association at the membrane
between the membrane molecule and the particular
membrane molecule indicator domain.

Exemplary properties of a membrane molecule
that can change in response to a stimulus, and which
10   can result in an increased or decreased association at
the membrane between the membrane molecule and the
MMID, include location (e.g. translocation of the
membrane molecule from its membrane location to a
different cellular location, or vice versa), abundance
15   (e.g. local, or overall, increase or decrease in
abundance of the membrane molecule at the membrane),
conformation (e.g. tertiary or quaternary structure,
which can reflect activation state), and post-
translational modification state (e.g. acylation,
20   biotinylation, mannosylation,, farnesylation,
formylation, geranyl-geranylation, hydroxylation,
methylation, myristoylation, palmitoylation,
phosphorylation, sulphation and the like). Therefore,
such properties of a membrane molecule, as indicated by
25   its relative ability to associate with a membrane
molecule indicator domain, reflect the presence and
nature of the stimulus. The appropriate property which
changes in response to a stimulus, will depend on the
nature of the membrane molecule and the stimulus.

30   As an example of a class of membrane
molecules that exhibit changes in properties in
response to stimuli, it is well known in the art that
tyrosine kinase receptors often exhibit changes in
location and abundance at the membrane (e.g. by

becoming internalized), conformation (e.g. by adopting
an activated tertiary conformation, dimerizing, or
associating with effector molecules), and/or post-
translational state (e.g. by becoming tyrosine
5   phosphorylated) in response to ligands. Certain
phospholipids exhibit changes in abundance (e.g. by
becoming hydrolyzed or produced) in response to agonist
activation of receptors. Other examples of membrane
molecules and changes in their properties in response
10   to stimuli, which can be detected using the methods and
compositions of the invention, are known in the art and
described further below.

     As used herein, the term "membrane," with
respect to the location of a membrane molecule detected
15   by the indicator compositions of the invention, refers
to any lipid bilayer of a cell, including, but not
limited to, the plasma membrane, Golgi membrane,
endoplasmic reticulum (ER) membrane, mitochondrial
membrane, endosomal membrane, peroxisomal membrane,
20   lysosomal or vacuolar membrane, and nuclear membrane.

     A membrane molecule can be of any nature,
such as a lipid, protein, saccharide, or any
combination thereof. In one embodiment, the membrane
molecule is a membrane lipid. Exemplary membrane
25   lipids include cholesterol, sphingolipids,
polyisoprenoids, mono-, di- and triacylglycerols, acyl
chains and their derivatives (e.g. arachadonic acid and
its metabolites, such as prostaglandins), and
phospholipids (e.g. phosphatidylcholine,
30   phosphatidylserine, phosphatidylethanolamine,
phosphatidic acid, phosphaytidlyglycerols, lyso-
derivatives thereof and phosphatidylinositols.
Exemplary phosphatidylinositols include $PtdIns(4,5)P_2$
(also referred to as PIP2), $PtdIns(3,4)P_2$,

15

PtdIns(3,4,5)P_3, PtdIns, PtdIns(3)P, PtdIns(4)P, as
well as D-enantiomers (e.g. D-Ins(1,4,5)P_3), di-carboxy
derivatives (e.g. DiC$_8$-PtdIns(4,5)P_2) and
glycerophosphoryl derivatives (e.g. g-PtdIns(4,5)P_2)of
5   these molecules.

The structural and regulatory function of
membrane lipids in normal and abnormal biological
processes, as well as the changes in properties of
lipids (e.g. abundance, localization, conformation and
10 post-translation modifications) that occur in response
to normal and pathological stimuli, are well known in
the art.

For example, a variety of sphingolipids have
roles in signaling, such as sphingosine in inhibiting
15 PKC, ceramide in modulating arachidonic acid (AA)
release, and sphingosine-1-phosphate in mobilizing
calcium (reviewed in Shayman, _Kidney International_
58:11-26 (2000). As other examples of the role of
membrane lipids in signaling, diacylglycerol (DAG)
20 activates protein kinase C (PKC); phosphatidic acid
(PA) activates certain kinases; and phosphatidyl
choline serves as a substrate for phospholipase D to
generate PA and then DAG, as well as a substrate for
phospholipase A2 to generate AA, which is the precursor
25 for eicosanoids and prostaglandins.

Phosphatidylinositols are particularly
important signaling molecules. For example, many cell
surface receptors are coupled to phospholipase C
activation. PLC activation cleaves the
30 phosphatidylinositol PIP2 to produce the second
messengers inositol 1,4,5-trisphosphate (IP3) and
diacylglycerol (DAG). These second messengers increase
intracellular Ca^{2+} concentration and activate the

16

serine/threonine specific protein kinase C (PKC),
respectively. PIP2 also serves as a substrate for
phosphatidyl inositol 3-kinase (PI3K), producing the
second messenger phosphatidylinositol (3,4,5)-
5   trisphosphate (PIP3). PIP2 also is implicated in the
regulation of the actin cytoskeleton, based on its
ability to bind to and regulate the function of a
number of actin severing, capping and bundling
proteins. Additionally, PIP2 modulates the activity of
10   phospholipase D (PLD), which catalyzes the hydrolysis
of phosphatidylcholine to phosphatidic acid and
choline.

       PIP2 resides at the plasma membrane of
resting cells. Upon agonist stimulation of a receptor
15   coupled to PLC, such as a tyrosine kinase receptor, or
a G-protein coupled receptor (GPCR) that acts through a
Gαq-containing effector G protein, PIP2 is hydrolyzed
to yield soluble IP3 and membrane bound DAG. PIP2 is
then resynthesized and returns to the membrane.
20   Accordingly, the abundance of PIP2 at the plasma
membrane reports the activation state of a PLC-coupled
receptor, in that high abundance of PIP2 at the plasma
membrane indicates the resting state, and low abundance
indicates agonistic activity through the receptor.

25   In an alternative embodiment, a membrane
molecule is a membrane protein. Exemplary membrane
proteins include integral membrane proteins such as ·
cell surface receptors (e.g. G-protein coupled
receptors (GPCRs), tyrosine kinase receptors, integrins
30   and the like) and ion channels; and proteins that
shuttle between the membrane and cytosol in response to
signaling (e.g. Ras, Rac, Raf, Gα subunits, arrestins,
Src and other effector proteins). In certain
embodiments, when specifically indicated, excluded from

the scope of the invention is a membrane molecule that
is a GPCR.

          The structural and regulatory function of
membrane proteins in normal and abnormal biological
5   processes, as well as the changes in their properties
(e.g. abundance, localization, conformation and post-
translation modifications) that occur in response to
normal and pathological stimuli, are well known in the
art.

10   As used herein, a "membrane molecule
indicator domain" or "MMID" refers to a domain that
associates with a membrane molecule with sufficient
affinity and selectivity to report a property of the
membrane molecule. The choice of membrane molecule
15   indicator domain will depend on the particular membrane
molecule. MMIDs for the membrane molecules described
above are known in the art, or can be readily
determined. Suitable MMIDs include, for example,
domains that mediate interaction with the membrane
20   molecule that are present in its naturally occurring
oligomeric partner(s), regulators and effectors, as
well as functional variants of such domains. Thus, for
example, MMIDs that bind to membrane molecules can
consist of SH2, SH3, PH, PTB, EH, PDZ, EVH1 and WW
25   domains that bind the membrane molecule *in vivo*, as
well as *functional variants of such domains.*

          In certain embodiments, such as when the
membrane molecule indicator is designed to indicate
activation state of a GPCR, the MMID can comprise a G-
30   protein subunit, such as a Gα, Gβ or Gγ subunit. For
example, high FRET between a Gα subunit linked to a
donor fluorescent domain and a Gβ and/or Gγ subunit
linked to an acceptor fluorescent domain (or vice

versa) can indicate the inactive state of the GPCR, in
which the trimeric G-protein complex is present at the
membrane. In contrast, low FRET can indicate
activation of the GPCR and dissociation of the G-

5   protein complex. In other embodiments, when
specifically indicated, excluded from the scope of the
invention is an MMID which comprises a G-protein
subunit.

        MMIDs also include domains which do not

10   normally interact with the membrane molecule in the
cell, but are determined, by methods known in the art,
to have sufficient affinity and selectivity to report a
property of the membrane molecule.

        Where the membrane molecule is a

15   phosphatidylinositol, a suitable membrane molecule
indicator domain is a phosphatidylinositol binding
domain. Phosphatidylinositol binding domains include,
for example, "pleckstrin homology" or "PH" domains,
"FYVE" domains, "C2" domains, "SH2" domains, PtdIns-

20   binding domains of actin-binding proteins, PtdIns-
binding domains of clathrin adaptor proteins, and START
domains (reviewed in Bottomley et al., Bioc. Biophys.
Acta 1436:165-183 (1998); Stenmark et al., J. Cell
Science 112:4175-4183 (1999); Janmey, Chem. Biol. 2:61-

25   65 (1995); and Ponting et al., TIBS 24:130-132
(1999)).

        In one embodiment, the phosphatidylinositol
indicator domain is a pleckstrin homology (PH) domain.
PH domains are generally around 120 amino acids long

30   and share characteristic structural features that
include two orthogonal β-sheets of three and four anti-
parallel β-strands, which sandwich an α-helix at the C-
terminus. PH domains also contain clusters of lysine
and arginine residues distal to the C-terminal α-helix

that create a highly charged surface, and an almost
invariant tryptophan residue near the C-terminus. PH
domains have been found in more than 100 different
proteins, including mammalian, *Drosophila*, *C. elegans*
5   and yeast proteins. Many PH domain containing proteins
are involved in intracellular signaling and
cytoskeletal organization.

Examples of PH domain containing proteins
include protein kinases (e.g. Btk, β-ARK and Akt), all
10   phospholipase C (PLC) isoforms (e.g. PLCβ, γ and δ),
insulin receptor substrates (IRS-1 and IRS-2),
phosphoinositide 3-kinase (PI3 kinase) p110γ subunit,
the guanine nucleotide release factor SOS, rasGAP,
dynamin, CDC25, Tiam-1, Vav, guanine nucleotide
15   exchange factors (e.g. GRP-1, ARNO, cytohesin) and β-
spectrin. The sequences, ligands and relative binding
affinities of a variety of PH domains are known in the
art (see, for example, Bottomley et al., <u>supra</u> (1998)).

A preferred PH domain is a PH domain of a
20   PLC, such as the PIP2-indicator PH domain of PLCδ1.
The cloning and expression of the PH domain of PLCδ1,   .
and its use in membrane molecule indicator
polypeptides, is described in the Example, below.

An alternative PH domain of a PLC is the PH
25   domain of PLCβ. PLCβ is responsible for physically
cleaving PIP2, and thus the PH domain therefrom can be
used to determine tranlocation or disassociation from
the membrane of the actual PIP2 lipids cleaved by PLCβ.
The PLCβ PH domain sequence is known in the art (e.g.
30   Rebecchi et al., <u>Annu. Rev. Biophys. Biomol. Struct</u>.
27:503-528 (1998)).

In another embodiment, the phosphatidylinositol indicator domain is an FYVE domain. FYVE domains have been demonstrated to specifically bind to PtdIns(3)P. FYVE domains

5   generally contain eight conserved cysteines, which coordinate two Zn^{2+} ions in a cross-braced topology, a conserved R(R/K)HHCRXCG (SEQ ID NO:1) motif surrounding the third and fourth cysteine residues, and several highly conserved hydrophobic residues (see, for

10 example, Stenmark et al., supra (1999), and Gaullier et al., Chem. Phys. Lipids 98:87-94 (1999)). FYVE domains have been found in mammalian, yeast and C. elegans proteins. Exemplary FYVE domain containing proteins include EEA1, Fab1p, YOTB, Vac1p, Vps27p, Hrs, Smad

15 anchor for receptor activation (SARA), Fgd1, and have also been described in a large number of proteins of unknown function whose sequences are available in public databases (Stenmark et al., supra (1999)).

In another embodiment, the phospholipid

20 indicator domain is a C2 domain. C2 domains are about 130 amino acids in length, and have been found in single or multiple copies in over 60 proteins. C2 domains bind a variety of ligands and substrates, including Ca^{2+}, phospholipids, inositol polyphosphates

25 and intracellular proteins. C2 domains are found, for example, in synaptotagmin I-VIII, rabphilin, phosphatidylserine decarboxylase, protein kinase C, GAPs, perforin, PLC family members, BCR, ABR, PI3-kinase, cytosolic phospholipase A2, and have also been

30 described in a large number of proteins of unknown function whose sequences are available in public databases (reviewed in Nalefski et al., Protein Science 5:2375-2390 (1996).

In yet another embodiment, the phospholipid indicator domain is an SH2 domain. SH2 domains are well-characterized mediators of protein-protein interactions, but in addition certain SH2 domains bind

5 phosphoinositides. For example, the SH2 domains from p85α and c-Src have been shown to directly and selectively bind PtdIns(3,4,5)P$_3$ (Bottomley et al., supra (1998)). The sequences of a variety of SH2 domains are known in the art.

10 In a further embodiment, the phospholipid indicator domain is a lipid binding domain of an actin binding protein, such as the lipid binding domain of the actin monomer sequestering protein profilin; the actin filament severing proteins gelsolin, villin,

15 severin, adseverin, destrin and cofilin; the protein gCap39, which blocks the ends of actin filaments; and the actin filament cross-linking protein α-actinin (reviewed in Janmey, supra (1995)). The sequences of a variety of lipid binding domains of actin binding

20 proteins are known in the art.

In another embodiment, the phospholipid indicator domain is a lipid binding domain of a clathrin adaptor protein, such as residues 5-80 of AP-2 (α-subunit), which specifically associates with

25 PtdIns(3,4,5)P$_3$, or residues 1-304 of AP-3, which specifically associates with pyrophosphate(PP)-InsP$_5$ (reviewed in Bottomley et al., supra (1998)). The sequences of a variety of lipid binding domains of clathrin adaptor proteins are known in the art.

30 Other membrane molecule indicator domains can be readily identified, for example, by database searching and by structural predictions based on

sequence or structural homology to known membrane
molecule indicator domains, as described above.

Association between a MMID and a membrane
molecule can be determined by binding assays known in
5   the art. For example, association can be determined by
co-immunoprecipitation assays, sedimentation assays,
affinity chromatography, two-hybrid assays, gel-overlay
assays, radiolabeled ligand binding assays, and the
like. Association between molecules can also be
10   determined by surface plasmon resonance (SPR) on
BIAcore, nuclear magnetic resonance (NMR) spectroscopy,
circular dichroism (CD) spectroscopy, and mass
spectroscopy. Association between a MMID and lipid
membrane molecule can conveniently be determined by
15   adsorbing the MMID to a vesicle containing the lipid,
and sedimenting the vesicle-bound protein by
centrifugation. Such methods are reviewed, for
example, in Winzor, <u>J. Mol. Recognit</u>. 13:279-298
(2000); and Bottomley et al., <u>supra</u> (1998).

20   The membrane molecule indicator domains
described herein need not have the exact sequence of a
domain found in a native sequence, so long as the
domain retains the membrane molecule indicator function
of the native sequence. Thus, a membrane molecule
25   indicator domain can be a variant sequence having one
or several amino acid additions, deletions or
substitutions compared with a native amino acid
sequence. Such modifications can be advantageous, for
example, in enhancing the stability, expression level,
30   or binding specificity of the domain, as well as for
facilitating chimeric polypeptide construction. The
function of a variant MMID can be confirmed by the
binding assays described above.

Modifications to the amino acid sequence of a
MMID can be randomly generated, such as by random
insertions, deletions or substitutions of nucleotides
in a native MMID nucleic acid molecule. Alternatively,
5 modifications can be directed, such as by site-directed
mutagenesis of a nucleic acid molecule encoding a
native MMID.

The skilled person appreciates that extensive
guidance in predicting which amino acid residues of a
10 MMID can be modified, while retaining membrane molecule
indicator ability, is provided by examining alignments
between orthologs and other members of a particular
MMID family. It is well known in the art that
evolutionarily conserved amino acid residues and motifs
15 are more likely to be important for maintaining
biological activity than less well-conserved residues
and domains. Thus, it would be expected that
substituting a residue that is highly conserved among
MMIDs within a family or across species with a non-
20 conserved residue may be deleterious, whereas making
the same substitution at a residue which varies widely
would likely not have a significant effect on
biological activity. These guiding principles have
been confirmed for a variety of MMID containing
25 proteins by mutagenesis studies. In general, a variant
MMID will have at least 70% identity, more preferably
at least 75% identity, including at least 80%, 85%,
90%, 95%, 98%, 99% or greater identity to the native
domain to which the variant domain is most closely
30 related.

Thus, as a non-limiting example, a PIP2
indicator domain can be a domain that has at least 70%
identity, more preferably at least 75% identity,
including at least 80%, 85%, 90%, 95%, 98% or greater

identity to amino acids 1-174 of the human PLCδ-1
sequence (GenBank Accession No. NM_006225).

As used herein, the term "membrane anchoring
domain" refers to the portion of a membrane molecule
5   indicator polypeptide that localizes the polypeptide to
a particular membrane. Membrane anchoring domains
suitable for localizing polypeptides to membranes of
interest are known in the art.

For example, a membrane anchoring domain
10   suitable for localizing a polypeptide to the plasma
membrane is the C-terminal sequence CaaX (where "a" is
an aliphatic residue, and "X" is any residue, generally
L). An exemplary membrane anchoring domain suitable
for localizing a polypeptide to the endoplasmic
15   reticulum is the C-terminal sequence KDEL (SEQ ID
NO:2), assuming a signal sequence present at the
N-terminus. Additionally, membrane anchoring domains
can be small proteins, and portions of proteins, that
confer appropriate localization to the membrane
20   molecule indicator polypeptide when present in a
chimera.

Optionally, the membrane anchoring domain can
be a second membrane molecule indicator domain that
associates with a different membrane molecule than the
25   first membrane molecule indicator domain, and that is
not co-regulated with the first membrane molecule. For
example, in order to determine membrane abundance of
PIP2, an appropriate indicator composition can include
a membrane molecule indicator domain that associates
30   with PIP2 (e.g. a PH domain) fused to a donor
fluorescent domain, and a membrane molecule indicator
domain that associates with a different membrane
molecule that is not co-regulated with PIP2 fused to an

25

acceptor domain, which thus serves to anchor the
acceptor domain to the plasma membrane.

        As used herein, the terms "donor fluorescent
domain" and "acceptor fluorescent domain" refer to a
5   pair of moieties selected so as to exhibit fluorescence
resonance energy transfer (FRET) when the donor moiety
is excited with appropriate electromagnetic radiation
or becomes luminescent.

        The donor fluorescent domain is excited by
10 light of appropriate intensity within its excitation
spectrum, and emits the absorbed energy as fluorescent
light. When the acceptor fluorescent domain is
positioned to quench the donor fluorescent domain in
the excited state, the fluorescence energy is
15 transferred to the acceptor fluorescent domain, which
can emit fluorescent light. FRET can be manifested as
a reduction in the intensity of the fluorescent signal
emitted from the donor fluorescent domain, by reduction
in the lifetime of the excited state of the donor
20 fluorescent domain, or by emission of fluorescent light
at the longer wavelengths (lower energies)
characteristic of the acceptor fluorescent domain.
When the association between the MMID and the
corresponding membrane molecule changes, the donor and
25 acceptor fluorescent domains physically separate (or
come closer together) , and FRET is decreased (or
increased) accordingly (see Figure 1).

        One factor to be considered in choosing the
fluorescent domain pair is the efficiency of
30 fluorescence resonance energy transfer between them.
Preferably, the efficiency of FRET between the donor
and acceptor moieties is at least 10%, more preferably
at least 50% and even more preferably at least 80%.

The efficiency of FRET can easily be empirically tested
using the methods described herein and known in the
art.

The efficiency and detectability of FRET also
5   depend on the separation distance and the orientation
of the donor and acceptor fluorescent domains, as well
as the choice of fluorescent domains. Considerations
for the choice of fluorescent domains are well known in
the art, and described, for example, in U.S. Patent
10   Nos. 5,998,204 and 5,981,200. For example, it is
preferred that the emission spectrum of the donor
fluorescent domain overlap as much as possible with the
excitation spectrum of the acceptor fluorescent domain.
In addition, the excitation spectra of the donor and
15   acceptor fluorescent domains should overlap as little
as possible so that a wavelength region can be found at
which the donor fluorescent domain can be excited
selectively and efficiently without directly exciting
the acceptor moiety. Likewise, the emission spectra of
20   the donor and acceptor fluorescent domains should have
minimal overlap so that the two emissions can be
distinguished. Furthermore, it is desirable that the
quantum yield of the donor fluorescent domain, the
extinction coefficient of the acceptor fluorescent
25   domain, and the quantum yield of the acceptor
fluorescent domain be as large as possible.

For example, in a suitable pair of
fluorescent domains, the donor fluorescent domain is
excited by ultraviolet light (<400 nm) and emits blue
30   light (<500 nm), while the acceptor fluorescent domain
is efficiently excited by blue light (but not by
ultraviolet light) and emits green light (>500 nm). In
an alternative pair of fluorescent domains, the donor
fluorescent domain is excited by violet light (about

400-430 nm) and emits blue-green light (450-500 nm),
while the acceptor fluorescent domain is efficiently
excited by blue-green light (but not by violet light)
and emits yellow-green light (about 520-530 nm).

5   Generally, the donor fluorescent domain and
acceptor fluorescent domain will be fluorescent
proteins, as described below. Alternatively, the donor
can contain a tag, such as an artificial tetracysteine-
based peptide tag, to which a cell permeable
10 fluorescent label, such as FLASH-EDT$_2$, can bind (e.g.
Griffin et al., Science 281:269-272 (1998)).

    Fluorescent proteins suitable for use as
donor or acceptor fluorescent domains in the
compositions and methods of the invention have been
15 isolated from a number of species, including jellyfish
(e.g. Aequorea species) and coral (e.g. Renilla species
and Discosoma species).

    In one embodiment, the donor and/or acceptor
fluorescent domain is a "green fluorescent protein" or
20 "GFP," such as a native GFP from an Aequorea or Renilla
species, an ortholog of a GFP from another genus, or a
variant of a native GFP with optimized properties. As
used herein, the term "GFP variant" is intended to
refer to polypeptides with at least about 70%, more
25 preferably at least 75% identity, including at least
80%, 90%, 95% or greater identity to a native GFP, such
as Aequorea victoria GFP.

    A variety of GFP variants having useful
excitation and emission spectra, have been engineered
30 by modifying the amino acid sequence of a naturally
occurring Aequorea or Renilla GFP (see, for example,

U.S. Pat. Nos. 5,625,048 and 5,998,204; Miyawaki et
al., <u>Nature</u> 388:882-887 (1997); Delagrave et al.,
<u>Biotechnology</u> 13:151-154 (1995); Pollok et al., <u>Trends
in Cell Biol</u>. 9:57-60 (1999)). Additionally, a variety
5   of enhanced GFPs (or EGFPs) with optimized codons for
expression in human cells, are known in the art (e.g.
ECFP and EYFP).

GFP variants with optimized dimerization
properties can also be prepared. It is postulated that
10 the weak dimerization observed between GFPs (e.g. kD
about 100 μM) allows donor and acceptor fluorescent
domains present on separate polypeptide chains (e.g.
Figure 1B or 1D) to associate at the membrane and
exhibit FRET, even at low expression levels where based
15 simply on polypeptide concentration at the membrane,
FRET would not be expected. The dimerization is
suitably weak so that once dissociated from the
membrane or from the membrane molecule, the donor and
acceptor fluorescent domains separate so as to no
20 longer exhibit FRET. GFP variants with altered
dimerization properties can be selected so as to
optimize the differential in FRET between alternatives
configurations. For example, GFP variants with
slightly higher, but still moderate, dimerization (e.g.
25 kD about 25 μM) are expected to provide for suitably
high FRET at the membrane even at low polypeptide
expression levels, while still separating once
dissociated from the membrane or from the membrane
molecule.

30   Cyan fluorescent proteins (CFPs) are variant
GFPs that contains the mutation Y66W with respect to
Aequorea victoria GFP. Yellow fluorescent proteins
(YFPs) are variant GFPs that contain aromatic residues
at position 203. Blue fluorescent proteins (BFPs) are

variant GFPs that contain a Y66H mutation. A group of
GFPs which lack the near-UV excitation peak, but retain
the wild-type GFP emission peak, have Ser65
substitutions. Other variants of native GFPs with
5 useful fluorescent properties are known in the art, or
can be readily prepared by random or directed
mutagenesis of a native GFP. Exemplary pairs of donor
and acceptor fluorescent domains include BFP-GFP and
CFP-YFP.

10   In another embodiment, the donor and/or
acceptor fluorescent domain is a "DsRed," such as a
native DsRed from a *Discosoma* species, an ortholog of
DsRed from another genus, or a variant of a native
DsRed with optimized properties (e.g. a K83M variant or
15 DsRed2 (available from Clontech)). As used herein, the
term "DsRed variant" is intended to refer to
polypeptides with at least about 70%, more preferably
at least 75% identity, including at least 80%, 90%, 95%
or greater identity to a native DsRed, such as a
20 *Discosoma* DsRed. Other variants of native DsReds with
useful fluorescent properties are known in the art, or
can be readily prepared by random or directed
mutagenesis of a native DsRed (see, for example,
Fradkov et al., FEBS Lett. 479:127-130 (2000)).

25   Other exemplary pairs of donor and acceptor
fluorescent domains, respectively, include GFP-dsRED2
and YFP-dsRED2.

          Included within the term "donor fluorescent
domain" is a bioluminescent domain, such as luciferase
30 from *Renilla*, related species, and variants thereof.
Renilla luciferase emits blue light in the presence of
an appropriate substrate, such as coelenterazin, which

can be transferred to an appropriate fluorescent
acceptor domain, such as a GFP, in a process called
Bioluminescence Resonance Energy Transfer, or BRET.
BRET is described, for example, in Angers et al., _Proc._
5 _Natl. Acad. Sci. USA_ 97:3684-3689 (2000); Xu et al.,
Proc. Natl. Acad. Sci. USA 96:151-156 (1999); and
components are commercially available from BioSignal
Packard (Montreal, Canada). Those skilled in the art
can readily apply the compositions and methods
10 described herein with respect to FRET, to compositions
and methods involving BRET.

In constructs in which the donor fluorescent
domain and the acceptor fluorescent domain are present
on the same polypeptide, the fluorescent domains can
15 optionally be separated by a flexible "linker
sequence." An appropriate linker sequence allows the
donor and acceptor fluorescent domain to be
functionally coupled when the single MMID (Figure 1A),
or pair of MMIDs (Figure 1B), are associated with a
20 membrane molecule, such that FRET is high, and
functionally uncoupled when the MMIDs are not
associated with the membrane molecule, such that FRET
is low (Figure 1D and E). In order to optimize the
FRET effect, the average distance between the donor and
25 acceptor fluorescent domains should become less than
about 10 nm when the MMID is associated with the
membrane molecule (e.g. from 1 nm to 10 nm).

The linker moiety preferably is between about
1 and 50 amino acid residues in length, preferably
30 between about 2 and 30 amino acid residues. A
preferred linker moiety contains, or consists of, the
sequence Gly-Gly, Ser-Gly or Gly-Ser. Linker moieties
and their applications are well known in the art and
described, for example, in U.S. Patent Nos. 5,998,204

and 5,981,200, and Newton et al., _Biochemistry_
35:545-553 (1996).

        The invention provides isolated nucleic acid
molecules, which alone or in combination as components
5   of a kit encode membrane molecule indicator
polypeptides, including each of the exemplary
indicators shown schematically in Figures 1, 9 and 10
and described above.

        As used herein, the term "nucleic acid
10   molecule" refers to a polynucleotide comprised of
either DNA or RNA; which can be single- or double-
stranded; which can optionally contain one or more non-
natural nucleotides, such as nucleotides having
modifications to the base, the sugar, or the phosphate
15   portion; and which can optionally contain one or more
non-natural linkages, such as phosphothioate linkages.

        As used herein, the term "kit" refers to two
or more component nucleic acid molecules packaged or
sold for use together. The kit components will be
20   contained either in a single container or separate
containers. The kit can further optionally contain
written instructions for use of the components in the
methods of the invention, and/or buffers and components
suitable for such methods.
25

        The invention nucleic acid molecules are
preferably operatively linked to a promoter of gene
expression. As used herein, the term "operatively
linked" is intended to mean that the nucleic acid
30   molecule is positioned with respect to either the
endogenous promoter, or a heterologous promoter, in
such a manner that the promoter will direct the

transcription of RNA using the nucleic acid molecule as
a template.

Methods for operatively linking a nucleic
acid to a heterologous promoter are well known in the
5   art and include, for example, cloning the nucleic acid
into a vector containing the desired promoter, or
appending the promoter to a nucleic acid sequence using
PCR. A nucleic acid molecule operatively linked to a
promoter of RNA transcription can be used to express
10 membrane molecule indicator transcripts and
polypeptides in a desired host cell or *in vitro*
transcription or transcription-translation system.

The choice of promoter to operatively link to
an invention nucleic acid molecule will depend on the
15 intended application, and can be determined by those
skilled in the art. For example, if the encoded
polypeptide may be detrimental to a particular host
cell, it may be desirable to link the invention nucleic
acid molecule to a regulated promoter, such that gene
20 expression can be turned on or off. Alternatively, it
may be preferred to have expression driven by either a
weak or strong constitutive promoter. Exemplary
promoters suitable for mammalian cell systems include,
for example, the SV40 early promoter, the
25 cytomegalovirus (CMV) promoter, the mouse mammary tumor
virus (MMTV) steroid-inducible promoter, and the
Moloney murine leukemia virus (MMLV) promoter.
Promoters suitable in yeast include, for example, ADH
promoter (*S. cerevisiae*) and the inducible Nmt *promoter*
30 (*S. pombe*).

It will be appreciated that a nucleic acid
molecule encoding a polypeptide containing a MMID and a

33

donor fluorescent domain, and a nucleic acid molecule
encoding a polypeptide containing a MMID and an
acceptor fluorescent domain (e.g. Figure 1B and 1D) can
optionally be present on the same vector or under the
5   control of the same promoter. Such constructs are
advantageous, for example, in simplifying introducing
the nucleic acid molecules into a cell and in ensuring
1:1 stoichiometry of the donor and acceptor in the
pair. Alternatively, the nucleotide sequences encoding
10 the two polypeptides can be present on separate vectors
or under the control of different promoters.

The invention further provides a vector
containing an isolated nucleic acid molecule encoding a
membrane molecule indicator polypeptide. Exemplary
15 vectors include vectors derived from a virus, such as a
bacteriophage, a baculovirus or a retrovirus, and
vectors derived from bacteria or a combination of
bacterial sequences and sequences from other organisms,
such as a cosmid or a plasmid. The vectors of the
20 invention will generally contain elements such as an
origin of replication compatible with the intended host
cells; one or more selectable markers compatible with
the intended host cells; and one or more multiple
cloning sites. The choice of particular elements to
25 include in a vector will depend on factors such as the
intended host cells; the insert size; whether regulated
expression of the inserted sequence is desired; the
desired copy number of the vector; the desired
selection system, and the like. The factors involved
30 in ensuring compatibility between a host cell and a
vector for different applications are well known in the
art.

For recombinant expression of the encoded
polypeptide, the isolated nucleic acid molecules will

generally be operatively linked to a promoter of gene expression, as described above, which may be present in the vector or in the inserted nucleic acid molecule.

5   Also provided are cells containing membrane · molecule indicators, including each of the exemplary indicators shown schematically in Figures 1, 9 and 10 and described above, and cells containing nucleic acid molecules encoding such indicators. The cells of the invention can advantageously express the encoded
10   polypeptide(s) and thus be used in screens for agonists, antagonists and inverse agonists of signaling pathways indicated by properties of the membrane . molecule; to functionally clone modulatory components of the signal transduction pathway in which the
15   membrane molecule is involved; and to determine or confirm the function of potential modulatory components of the signal transduction pathway in which the membrane molecule is involved. Such applications are described further below.

20   The isolated nucleic acid molecule(s) will generally be contained within an expression vector, but optionally can be expressible DNA or RNA not contained within a vector. The isolated nucleic acid molecule(s) can be maintained episomally, or incorporated into the
25   host cell genome. The cells of the invention can be prepared by introducing the nucleic acid molecules of the invention by any suitable means, including, for example, transfection, transduction, electroporation and microinjection, as well as by transgenic
30   technology.

The cells of the invention can be prepared from any organism, including, for example, bacteria (e.g. *E. coli*), insects (e.g. *Drosophila*), yeast (e.g.

S. cerevisiae, S. pombe, or *Pichia pastoris*), nematodes
(e.g. *C. elegans*), amphibians (e.g. *Xenopus* embryos and
oocytes) and mammals (e.g. human, rodent or primate
primary cells and established cell lines, such as COS,
5   CHO, 3T3, N1E-115, HEK, etc., representing either a
normal or diseased state of the mammal).

The cells of the invention can further
recombinantly express, either stably or transiently, a
known or candidate modulator of the membrane molecule,
10   such as a known or candidate agonist, antagonist or
reverse agonist peptide; a known or candidate receptor;
or a known or candidate effector molecule.

As used herein, the term "recombinant
expression," with respect to expression of a signaling
15   polypeptide, refers to transient or stable expression
of a polypeptide from a recombinant nucleic acid
molecule. Recombinant expression is advantageous in
providing a higher level of expression of the
polypeptide than is found endogenously, and also allows
20   expression in cells or systems in which the polypeptide
is not normally found.

The term "recombinant nucleic acid molecule"
is intended to refer to a nucleic acid molecule that
25   has been constructed, at least in part, by molecular
biological methods, such as PCR, restriction digestion
or ligation. A recombinant nucleic acid expression
construct generally will contain a constitutive or
inducible promoter of RNA transcription appropriate for
30   the host cell or transcription-translation system,
operatively linked to a nucleotide sequence that
encodes the polypeptide of interest. The expression
construct can be DNA or RNA, and optionally can be

36

contained in a vector, such as a plasmid or viral
vector.

        The construction of expression vectors and
the expression of genes in transfected cells involves
5   the use of molecular cloning techniques well known in
the art and described, for example, in Sambrook et al.,
Molecular Cloning--A Laboratory Manual, Cold Spring
Harbor Laboratory, Cold Spring Harbor, N.Y., (1989) and
Current Protocols in Molecular Biology, F. M. Ausubel
10 et al., eds., (Current Protocols, a joint venture
between Greene Publishing Associates, Inc. and John
Wiley & Sons, Inc., (most recent Supplement).

        The nucleotide sequences of various receptors
and effectors, and methods of recombinantly expressing
15 the encoded polypeptides in a variety of cell types,
are well known in the art.

        The invention further provides membrane
molecule indicator polypeptides. The invention
polypeptides include polypeptides recombinantly
20 expressed by the invention nucleic acid molecules, as
well as constructs produced by chemically coupling some
or all of the component domains, which themselves can
be recombinantly produced. The expressed polypeptides
can optionally be isolated from a transcription-
25 translation system or cell, by biochemical and
immunological purification methods known in the art.
To facilitate isolation, the invention polypeptides can
optionally be fused to a tag sequence, such as an
epitope tag, a GST polypeptide or a 6XHis fusion.

30   The invention polypeptides can optionally be
introduced into a whole cell, such as by recombinant
expression or microinjection, and the cells used in the

methods described herein. The invention polypeptides
can alternatively be introduced into a lipid bilayer,
such as a cellular membrane extract, or an artificial
lipid bilayer (e.g. a liposome vesicle). Methods of
5 preparing lipid bilayers containing desired amounts and
types of molecules, including the membrane molecules
and MMID polypeptides described herein, are known in
the art.

10   The invention also provides a method of
determining a property of a membrane molecule. The
method is practiced by
          (a) providing a cell or lipid bilayer comprising a
membrane molecule indicator; and
15   (b) determining FRET between the donor fluorescent
domain and the acceptor fluorescent domain,
          wherein FRET between the donor domain and the
acceptor domain is indicative of a property of the
membrane molecule.

20   Also provided is a method of identifying a
compound that modulates a property of a membrane
molecule. The method is practiced by
          (a) contacting a cell or lipid bilayer comprising
a membrane molecule indicator with one or more test
25 compounds, wherein the cell or bilayer further
comprises said membrane molecule; and
          (b) determining FRET between the donor fluorescent
domain and the acceptor fluorescent domain following
contacting,
30   wherein increased or decreased FRET following
contacting indicates that the test compound is a
compound that modulates a property of the membrane
molecule.

The lipid bilayer useful in such methods can
be a whole cell, which naturally or recombinantly
expresses the membrane molecule, or a cellular extract
containing the plasma membrane or vesicular membranes.

5   Alternatively, the lipid bilayer can be a lipid
vesicle, which can include either natural or synthetic
lipids or both, into which a membrane molecule of
interest is incorporated. Lipid vesicles are
advantageous in that the abundance of the membrane

10 molecule (and, optionally, of other signaling molecules
of interest) can be controlled.

The methods of the invention are useful in
the practice of essentially any application for which a
readout of signal transduction mediated through

15 membrane molecules is useful. Such applications are
well known in the. art.

Exemplary applications include 1) identifying
test compounds that act as agonists, antagonists,
inverse agonists or natural ligands of receptors

20 (described further below); 2) expression cloning of
peptide agonists, antagonists and inverse agonists of
receptors; 3) expression cloning of novel modulators
that affect the abundance, localization, conformation
or post-translational modification state of the

25 membrane molecule of interest (e.g. enzymes, enzyme
inhibitors, transcriptional regulators, and the like),
which themselves can be used as therapeutic drug
targets; 4) determining the function of variants of
known or predicted modulators of membrane molecules

30 (e.g. determining the effect of SNPs, disease-
associated mutations and engineered variations in
receptors, effectors and the like); 5) establishing
dose-response curves of modulators of membrane
molecules (e.g. for predicting effective dose of a

39

therapeutic); and 6) determining alterations in
membrane molecules and modulators that reflect disease
state, which can be applied to the development of
diagnostic methods. Methods of using the compositions
5   and methods described herein for such applications, and
other applications relating to signal transduction,
will be readily apparent to the skilled person.

As an example, the methods of the invention
10 can be used to identify test compounds that are
agonists, antagonists, inverse agonists or natural
ligands of receptors, including G-protein coupled
receptors (described further below), tyrosine kinase
receptors (e.g. PDGF, IGF, FGF and EGF receptors and
15 the like) and integrins. In the methods of the
invention, the basal level of FRET can be determined in
an unstimulated lipid bilayer. The lipid bilayers can
then be contacted with a test compound, and FRET
compared with an unstimulated bilayer. FRET is
20 advantageous over fluorescent visualization methods in
that both increases and decreases, relative to the
basal level, can be readily determined. Increased or
decreased FRET relative to the basal level is a
reflection of the activity of the test compound as an
25 agonist, antagonist (or inverse agonist) of the
signaling pathway linked to the membrane molecule.

As used herein, the term "agonist" refers to
a molecule that selectively activates or increases
signal transduction. An agonist can act by any
30 mechanism, such as by binding a receptor at the normal
ligand binding site, thereby mimicking the natural
ligand and promoting receptor signaling. An agonist
can also act, for example, by potentiating the binding
ability of the natural ligand, or by favorably altering
35 the conformation of the receptor. The compositions and

methods of the invention can advantageously be used to
identify agonists that acts through any agonistic
mechanism.

5   As used herein, the term "antagonist" refers
to a compound that selectively inhibits or decreases
signal transduction. An important subset of antagonist
compounds that can advantageously be identified by the
methods described herein, are referred to as "inverse
agonists." Inverse agonists are antagonists that
10   selectively inhibit or decrease signal transduction
below basal levels.

        An antagonist can act by any antagonistic
mechanism, such as by binding to a ligand or receptor,
thereby inhibiting their interaction. An antagonist
15   can also act by modifying or altering the native
conformation of a receptor. The methods of the
invention can advantageously be used to identify an
antagonist that acts through any antagonistic
mechanism.

20   For therapeutic applications, an agonist
preferably has an EC_{50}, and an antagonist or inverse
agonist preferably has an IC_{50}, of less than about 10^{-7}
M, such as less than 10^{-8} M, and more preferably less
than 10^{-9} M. However, depending on the stability,
25   selectivity and toxicity of the compound, an agonist
with a higher EC_{50}, or an antagonist with a higher IC_{50},
can also be useful therapeutically. EC_{50} and IC_{50} of
such compounds can be established by dose-response
curves using the methods described herein.

30   As used herein, the term "test compound"
refers to any molecule that potentially acts as an
agonist, antagonist, inverse agonist or natural ligand

41

of a signaling pathway reported by the membrane
molecule indicator compositions and methods of the
invention. A test compound can be a naturally
occurring macromolecule, such as a polypeptide, nucleic

5   acid, carbohydrate, lipid, or any combination thereof.
A test compound also can be a partially or completely
synthetic derivative, analog or mimetic of such a
macromolecule, or a small organic molecule prepared by
combinatorial chemistry methods.

10   Methods for preparing large libraries of
compounds, including simple or complex organic
molecules, metal-containing compounds, carbohydrates,
peptides, proteins, peptidomimetics, glycoproteins,
lipoproteins, nucleic acids, antibodies, and the like,

15   are well known in the art and are described, for
example, in Huse, U.S. Patent No. 5,264,563; Francis et
al., Curr. Opin. Chem. Biol. 2:422-428 (1998); Tietze
et al., Curr. Biol., 2:363-371 (1998); Sofia, Mol.
Divers. 3:75-94 (1998); Eichler et al., Med. Res. Rev.

20   15:481-496 (1995); and the like. Libraries containing
large numbers of natural and synthetic compounds also
can be obtained from commercial sources.

          The number of different test compounds to
assay in the methods of the invention will depend on

25   the application of the method. For example, one or a
small number of test compounds can be advantageous in
manual screening procedures, or when it is desired to
compare efficacy among several predicted ligands,
agonists or antagonists. However, it is generally

30   understood that the larger the number of test
compounds, the greater the likelihood of identifying a
compound having the desired activity in a screening
assay. Additionally, large numbers of compounds can be
processed in high-throughput automated screening

assays. Therefore, "one or more test compounds" can
be, for example, 2 or more, such as 5, 10, 15, 20, 50
or 100 or more different compounds, such as greater
than about 10^3, 10^5 or 10^7 different compounds.

5   A lipid bilayer can be contacted with a test
compound by any mode, such as by extracellular
administration, by intracellular uptake of the
compound, or by recombinant expression of the compound
(in methods involving an intact cell). The contacting
10   with the test compound can optionally take place in the
presence of a known agonist or antagonist of the
signaling pathway of interest, and the effect of the
compound on agonist or antagonist-mediated signaling
can be assessed.

15   In cases in which the test compound is a
peptide, the peptide can preferentially be targeted to
the membrane location of the membrane molecule of
interest, such as the extracellular or intracellular
face of the plasma membrane, Golgi, ER or the like,
20   using known methods. For example, test compounds that
are peptides can be expressed on the extracellular
membrane of a cell or the invention, or on a second
cell, by phage display methods known in the art.
Alternatively, test compounds that are peptides can be
25   secreted from a cell of the invention, or from a second
cell, by expressing the peptide with a secretory
signal.

          As an example, the methods of the invention
can be used to screen for G-protein coupled receptor
30   (GPCR) agonists, antagonists and inverse agonists, as
well as to identify the natural ligands of orphan
GPCRs.

GPCRs are seven-transmembrane-domain
polypeptides that transduce G-protein coupled signals
in response to ligands. The natural agonists of
different GPCRs range from peptide and non-peptide
5   neurotransmitters, hormones and growth factors, to
lipids, nucleoside-sugars, amino acids, light and
odorants. GPCRs are involved in a variety of critical
biological functions, including cell proliferation,
differentiation and apoptosis. GPCRs have proven to be
10   important targets of pharmaceuticals that affect a
variety of diseases, including neurological and
psychiatric disorders, vascular diseases,
endocrinological disorders, and cancer. It is
estimated that over 50% of current drugs are targeted
15   towards GPCRs, and represent about a quarter of the 100
top-selling drugs worldwide.

The natural ligands of different GPCRs
include peptides, biogenic amines, glycoproteins,
nucleotides, ions, lipids, amino acids, light and
20   odorants. Structurally, GPCRs can be divided into
three major subfamilies, each of which currently
includes orphan receptors as well as receptors whose
ligands are characterized (reviewed in Gether,
Endocrine Reviews 21:90-113 (2000)). A database
25   containing links to the nucleotide and amino acid
sequences of numerous mammalian GPCRs, including orphan
GPCRs, is available at
http://www.darmstadt.gmd.de/~gpcrdb/.

As used herein, the term "G-protein" refers
30   to a class of heterotrimeric GTP binding proteins, with
subunits designated Gα, Gβ and Gγ, that couple to
seven-transmembrane cell surface receptors to couple
extracellular stimuli to intracellular messenger
molecules. G-proteins are distinguished by their Gα

44

subunits. The more than 20 different Gα subunits,
encoded by 17 different genes, can be grouped into four
major families: Gαs, Gαi, Gαq, and Gα12. Signaling
through GPCRs that couple to Gαq-containing G proteins

5   activates PLC enzymes to hydrolyze PIP2 in the plasma
membrane to DAG and IP3.

    The specificity of Gα subunits for GPCRs is
determined by the C-terminal five amino acids of the
Gα. Thus, a variety of signal transduction pathways

10   can be assayed to determine signaling through a GPCR,
by co-expressing a chimeric Gα containing the five C-
terminal residues of a Gα known or predicted to couple
to the receptor of interest (such as Gαi, Gαs or the
promiscuous Gα16), with the remainder of the protein

15   corresponding to a Gα coupled to the GPCR that signals
through a membrane molecule of interest (see Conklin et
al., Nature 363:274-276 (1993), and Komatsuzaki et al.,
FEBS Letters 406:165-170 (1995)).

    For example, in instances in which the

20   membrane molecule indicator polypeptides are designed
to indicate abundance of PIP2, cells (or other lipid
bilayers) can contain a GPCR of interest, and
optionally a Gαq or Gα16 (or chimeric or variant Gα
which functions as a Gαq). The basal level of FRET

25   between acceptor and donor fluorescent domains linked
to a MMID (or two MMIDs) that associate with PIP2 can
be determined. In response to agonist-induced signal
transduction through the GPCR, PIP2 is hydrolyzed and
FRET is decreased, as exemplified in the cells

30   described in the Example, below. Likewise,
antagonistic or inverse agonistic effects can be
determined by an increase in agonist-induced, or basal,
levels of FRET.

In the cells described in the Example, below,
in which the MMID associates with PIP2 in the plasma
membrane, FRET is high in unstimulated cells. In the
presence of a test compound that activates PLC (e.g.
5   bradykinin), FRET is significantly lower than in
unstimulated cells, as PIP2 in the membrane is
hydrolyzed, and the donor and acceptor fluorescent
domains are no longer in close proximity. Thus, the
compositions and methods described in the Example,
10 below, can be used to identify and compare test
compounds that stimulate the activation of PLC, that
decrease the basal level of PLC activation, or that
antagonize agonist-induced PLC activation.

PIP2 hydrolysis leads to the production of
15 the second messengers DAG and IP3. IP3 mediates the
release of Ca^{2+} from intracellular stores. Ca^{2+} release
has been used as a signaling assay in variety of
research, diagnostic and screening applications. The
methods described herein, which detect PIP2 hydrolysis,
20 can be used in most applications in which determination
of Ca^{2+} release has proven useful.

As disclosed herein, determining PIP2
hydrolysis has certain advantages over determining Ca^{2+}
release, in that PIP2 hydrolysis is more proximal to
25 receptor activation, and is thus less dependent on
intermediate signaling steps that may introduce
variability. As shown herein, signals that yield
similar Ca^{2+} responses have different PLC activation
kinetics, suggesting that PIP2 hydrolysis follows
30 receptor activation more faithfully than Ca^{2+}
responses.

Optionally, PIP2 hydrolysis, as determined by
the FRET methods described herein, and Ca^{2+} release, as

determined using Ca^{2+} indicator dyes known in the art,
can both be assayed. Because Ca^{2+} release is
downstream of PIP2 hydrolysis, Ca^{2+} release can be
assayed simultaneously with FRET to confirm that the
5   observed FRET reflects PIP2 hydrolysis.

Methods of determining and quantitating FRET
at the single cell level, or in cell populations, are
well known in the art or can be determined by the
skilled person. For example, FRET can be measured
10 using dual emission fluorescence microscopy, as
described in the Example, below. Alternatively, FRET
can be measured using fluorescent microscopy imaging
methodology, which allows for simultaneous recordings
from multiple cells.

15   As a further example, FRET can be determined
with fluorescent lifetime. Briefly, upon excitation
with an ultrashort pulse of light (e.g. about 0.01 ns),
fluorophores have a characteristic decay in emission
that is single exponential, and may last 0.1-10ns,
20 dependent on the fluorophore and conditions. It has
been shown that the presence of a FRET acceptor
dramatically shortens the decay time of the donor,
which can be detected either using direct monitoring of
the decay time (time domain monitoring), or using
25 sine-modulated light, in the frequency domain (see, for
example, Verveer et al., Biophys. J., 78:2127-37
(2000)).

For high-throughput screening applications,
FRET can be measured using fluorescence activated cell
30 sorting (FACS), such as with a HeCd laser or frequency-
double diode laser. FACS is advantageous in permitting
the analysis of around 50,000 cells per second, which
is orders of magnitude faster than visual detection

47

methods. FACS also allows the isolation of cells for further growth, manipulation and identification of nucleic acid molecules encoding compounds that modulate association between membrane molecules and the MMIDs of

5   the invention compositions.

Therefore, the compositions and methods of the invention are amenable to high-throughput screening for potential therapeutics.

10   The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Preparation and Use of Membrane Molecule
Indicator Compositions

15   This example shows the preparation of two pairs of nucleic acid molecules of the invention. In the first pair, the first nucleic acid molecule encodes a polypeptide containing a membrane molecule indicator domain (PH domain) and a donor fluorescent domain

20   (CFP), and the second nucleic acid molecule encodes a polypeptide containing a membrane molecule indicator domain (PH domain) and an acceptor fluorescent domain (YFP). In the second pair, the first nucleic acid molecule encodes a polypeptide containing a membrane

25   molecule indicator domain (PH domain) and a donor fluorescent domain (CFP), and the second nucleic acid molecule encodes a polypeptide containing a membrane anchoring domain (CaaX) and an acceptor fluorescent domain (YFP).

30   This example also shows the use of the pairs of nucleic acid molecules to determine the abundance of

48

a membrane molecule (PIP2), by determining FRET between
the donor and acceptor fluorescent domains. High FRET
results from high PIP2 abundance at the plasma
membrane, which indicates the resting state of the
5 cell; decreased FRET results from PIP2 hydrolysis,
which indicates signaling through a G-protein coupled
receptor linked to PLC activation.

Experimental Procedures

Materials

10   1-oleoyl LPA, histamine, bradykinin (BK),
phenyl-arsine oxide and quercetin were from Sigma
Chemical Co. (St. Louis, MO); neurokinin A, caged IP3
(cat.# 407135) and ionomycin were from
Calbiochem-Novabiochem Corp. (La Jolla, CA);
15 Myo-[^{3}H]inositol (60 Ci/mmol) was from
Amersham-Pharmacia Biotech. Fura-red (K salt) was from
Molecular Probes Inc. (Eugene, OR). All other
chemicals were of analytical grade.

Constructs

20   The pleckstrin homology-domain of human
phospholipase C δ1 was obtained from the
Superhiro-PLCδ1 PH construct (AA 1-174, obtained from
T. Meyer) and cloned into the eukaryotic expression
vector pECFP-C1 (Clontech, CA). Two primers (PLCδPH1;
25 5' CCTGC GGCCG CGGTA CCGAT ATCAG ATGTT GAGCT CCTTC AC
3' (SEQ ID NO:3) and PLCδPH2; 5' CCGAA TTCCC GGGTC
TCAGC CATGG ACTCG GCCGG GACT TC 3'(SEQ ID NO:4)) were
designed to generate the PH-domain in frame behind the
CFP followed by a stop codon. The PCR-product was
30 cloned into the pECFP plasmid with the restriction

sites EcoRI and EcoRV on EcoRI and SmaI, leading to
pECFP-PH.

YFP was obtained from yellow Cameleon 2.0
(obtained from A. Miyawaki and R. Tsien) and subcloned
5   into cloning vector PGEM3z (Promega), via SacI and
EcoRI, and subsequently into pcDNA3 (Invitrogen) via
BamHI and EcoRI. PCR on YFP- pcDNA-3 with primers T7
(Promega) and GFP3; 5' GGCTG AGACC CGGGA ATTCG GCTTG
TACAG CTCGT CCATG 3' (SEQ ID NO:5) was done to remove
10 the stop codon. The PH domain PCR-product, taken
between primers PLCδPH1 and PLCδPH2, was cloned in
frame behind YFP with EcoRI and NotI, leading to
pcDNA3YFPPH. To obtain pcDNA3eGFPPH, YFP was swapped
with EGFP, using primers T7 and GFP3 on pcDNA3eGFP and
15 restriction enzymes BamHI and EcoRI.

For YFP-CAAX and GFP-CAAX, the membrane
localization sequence of K-Ras (KMSKD GKKKK KKSKT
KCVIM; SEQ ID NO:6) was obtained by PCR from Bp180-CAAX
(GenBank accession number M54968 and M38506), using
20 primers CAAX3 5'CCGAA TTCCC GGGTC AAGAT GAGCA AAGAT
GGTAA AAAG 3' (SEQ ID NO:7), containing an EcoRI site,
and CAAX2; 5' CCTGC GGCCG CGGTA CCGAG ATCTT TACAT AATTA
CACAC TT 3' (SEQ ID NO:8), that contained a NotI-site
behind the stop codon. The final constructs were made
25 by exchanging the PH domain from YFP-PH and GFP-PH for
the CAAX domain using EcoRI and NotI. All clones were
verified by sequence analysis. YFP-CAAX contained a
point mutation (V instead of G in the CAAX domain), but
this did not influence the membrane localization.

30   Constitutively active mutants of Gα$_q$ and Gα$_{12}$
subunits in pcDNA3 vectors were obtained from Dr. O.
Kranenburg (Kranenburg et al., Mol. Biol. Cell
10:1851-1857 (1999)).

50

Cell culture and transfections

     N1E-115 neuroblastoma cells were seeded in
6-well plates at about 25,000 cells per well on 25mm
glass coverslips, and cultured in 3 ml Dulbecco's
5   Modified Eagle's medium (DMEM) supplemented with 10%
FCS and antibiotics. Unless otherwise indicated,
constructs were transfected for 6-12 hours using
calcium phosphate precipitate, at 0.8 µg DNA/well.
Following transfection, cells were incubated in serum-
10   free DMEM for 12-48 hours. For fluorescence
detections, coverslips with cells were transferred to a
culture chamber and mounted on an inverted microscope.
All experiments were performed in bicarbonate-buffered
saline (containing, in mM, 140
15   NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, with 10mM
HEPES added), pH 7.2, kept under 5% CO_2, at 37°C.

Inositol phosphate determinations

     Preparation, culture and labeling of bovine
adrenal glomerulosa cells have been described in Balla
20   et al., J. Biol. Chem. 269:16101-16107 (1994). Cells
labeled with myo-[^{3}H]inositol for 24-48 hrs were
stimulated by angiotensin II (30 nM) for the indicated
times in a medium containing either Sr^{2+} or Ca^{2+}.
Reactions were terminated with perchloric acid and
25   inositol phosphates were separated by HPLC essentially
as described in Balla et al., supra (1994).

Confocal microscopy and image analysis

     For confocal imaging, a Leica DM-IRBE
inverted microscope fitted with TCS-SP scanhead was
30   used. Excitation of EGFP was with the 488 nm Argon ion
laserline, and emission was collected at 500-565 nm.

51

For translocation studies, a series of confocal images
were taken at 2-10 second intervals and stored on disk.
Determination of the ratio of membrane to cytosolic
fluorescence by directly assigning regions of interest
5   (ROIs) for membrane and cytosol was hampered by the
shape changes of cells during experiments. Using Qwin
software (Leica) this ratio was therefore calculated by
post-acquisition automated ROI assignment and analysis.
In brief, a binary mask of the transfected cell was
10 lined out using a thresholding step on a smoothed
image. From this mask, the area corresponding to the
membrane was eroded by a selectable amount to delineate
the membrane. Further erosion was then applied to
reliably separate membrane from cytosol area, and the
15 remaining area was taken to represent cytosol. This
mask was updated for each image in a series, and
translocation was expressed as ratio of the
fluorescence values for membrane and cytosol area, to
correct for bleaching. This approach corrects fully
20 for cell movements and shape changes, and was able to
reliably detect very minor translocations (using e.g.
diluted agonists).

Fluorescence determinations

For FRET experiments, cells were transferred
25 to an inverted Zeiss Axiovert 135 microscope equipped
with dry Achroplan 63x (NA 0.75) objective. Excitation
of CFP was at 425±5 nm, and emission was collected with
a 460 nm dichroic mirror. Emission of CFP and YFP was
split using an additional 505 dichroic and filtered
30 with 475DF30 and 540DF40 bandpass filters,
respectively. Detection was with PTI model 612 analog
photomultipliers, and for data acquisition, the FELIX
software (PTI Inc.) was used. FRET was expressed as
the ratio of CFP to YFP signals, the value of which was

set as 1.0 at the onset of the experiment. Changes are
expressed as percent deviation from this initial value
of 1.0.   For detection of intracellular Ca^{2+}, Yellow
Cameleon 2.1 was used at the same wavelengths (Miyawaki
5   et al., _Nature_ 388: 882-887 (1997)).

        For sustained stimulation, agonists and
inhibitors were added to the medium from concentrated
stocks. Stimulation with short pulses of NKA was
performed by placing a glass micropipette (tip
10 diamenter about 2 µm) at about 25 µm from the cell
using an Eppendorf microinjection system and applying
pulses of pressure for 10 seconds. It was verified
using Lucifer Yellow in the pipette that following
termination of the pressure pulse the concentration at
15 the cell rapidly dropped towards zero.

Loading and flash Photolysis of Caged IP3

        Before electroporation, adherent cells grown
on coverslips were washed twice in intracellular buffer
(containing in mM: 70 KCl, 70 Kglutamate, 2 $MgCl_2$, 0
20 $CaCl_2$, 5 phosphate buffer, pH 7.1) and then 70 µl of
this intracellular buffer was added to the cells with
20 µM of Fura-red tetrapotassium salt and either 1, 10
or 100 µM of caged IP3. Electroporation was achieved
by a series of 15 high-frequency square wave pulses,
25 (1-second spaced, amplitude 150V, frequency 80 kHz,
lasting 0.5 ms each) using 2 platina electrodes of 8 x
3mm with 2.5 mm spacing. The efficiency of this method
was assessed by control permeabilizations that were
performed on the stage of a confocal microscope. This
30 protocol caused complete permeabilization (based on
equilibration of intracellular calcein concentrations
with the extracellular buffer) of the cells in the area
between the electrodes.

For photorelease of caged IP3, a single cell
was illuminated with a short pulse of UV light (340-410
nm) from a 100W HBO lamp using a shutter. The shutter
open time was adjusted to give full release of caged
5   IP3, that is no response being observed with a
subsequent illumination. For partial photolysis, the
flash intensity was adjusted by using neutral density
filters placed in the illumination pathway.

10   Quantitation of expression levels

For quantitation of expression levels of
CFP-PH and YFP-PH, cellular fluorescence was compared
to the fluorescence of a solution of known
concentration of purified, bacterially expressed CFP-PH
15   or YFP-PH, following the method of Miyawaki et al.,
"Calcium signaling: a practical approach," Oxford
University Press (in press). In short, CFP-PH and
YFP-PH were expressed as GST-fusion proteins, and
purified on glutathione sepharose beads. Protein
20   concentration was measured by the BCA* Protein Assay
(Pierce, Illinois, USA). The solution (4.8 µM) was
then introduced in a linear wedge-shaped chamber (0-170
µM thickness) that was placed on the microscope (using
NA 0.7 objective), and the position of the chamber was
25   adjusted to give a fluorescence readout that matched
that of a single, CFP or YFP expressing cell. The
estimate of the fluorescent protein concentration in
the cell was obtained by comparing the local thickness
of the wedge to that of an average cell (17 µM).
30   Relative amounts of CFP-PH and YFP-PH expression in
cells were always determined under conditions of full
cytosolic localization of the constructs.

At the onset of each experiment,
photomultiplier gains (high voltage) were adjusted to
give a standard 6V output for the resting cell. Noting
that over an extended range of light input, every
5   2-fold change in intensity corresponds to a 35V change
in cathode voltage, cell intensities were measured. By
comparing these to the values obtained with the
GFPwedge, estimates of expression levels were obtained.

Fluorescence Recovery After Photobleaching (FRAP)

10   For FRAP experiments, cells were imaged using
a Leica TCS-SP confocal microscope equipped with 63x
(NA 1.3) oil immersion objective. The beam from an
external ArKr laser (25 mW) was coupled into the
backfocal plane of the objective via the
15   epifluorescence excitation port, using a 30/70
beamsplitter, thus allowing simultaneous imaging and
spot bleaching. Spots of about 1.3 μm (full width half
maximum) were bleached (>95%) in the basal membrane
using a single 30 ms pulse from the ArKr laser during
20   data collection in linescan mode at 1000, 500 or 125
Hz. Data were corrected for slight (<7%) background
bleaching and fitted with single exponents using
Clampfit software (Axon Instruments, CA).

Results

25   Fluorescence Resonance Energy Transfer between plasma
membrane-localized PLCδ1PH-CFP and PLCδ1PH-YFP

          PH-CFP and PH-YFP chimera were transiently
transfected into N1E- 115 mouse neuroblastoma cells at
a 1:1 molar ratio. After 1-2 days, cells were
30   transferred to an inverted epifluorescence microscope
and assayed for FRET by simultaneously monitoring the

emission of CFP (475±15 nm) and of YFP (530±20 nm),
while exciting CFP at 425±5 nm. In resting cells,
PH-CFP and PH-YFP reside at the plasma membrane bound
to PI(4,5)P2, and the two fluoriphores remain within

5   resonance distance. Upon activation of PLC by the
addition of bradykinin (BK), PI(4,5)P2 is rapidly
hydrolyzed and consequently PH domains can no longer
bind to the plasma membrane. Depending on cell type
(surface to volume ratio), it is estimated that the

10 distance(s) between fluoriphores increase about
200-1000 fold), and therefore FRET does not occur (Fig.
2). As a result, the donor (CFP) emission intensity
increases, while the acceptor (YFP) emission decreases.
By taking the ratio of CFP to YFP emission, the FRET

15 signal becomes essentially independent on excitation
intensity fluctuations and photobleaching..

The kinetics of BK-induced PLC activation in
N1E-115 cells as detected by FRET is characterized by a
rapid onset, with translocation peaking at 20-30 s

20 after addition of the agonist. The decaying phase is
somewhat slower, usually returning to baseline within 1
to 4 minutes. This time course is very similar to that
deduced from confocal detection of PLCδ1PH-GFP
translocation recorded under identical conditions (Fig.

25 2C). In this latter case, the data were extracted from
a time series using post-acquisition automated image
analysis (see Experimental Procedures). Similar
translocation responses can be obtained by FRET in
other cell types, including A431 epidermoid carcinoma

30 cells, HEK293 embryonal kidney cells, and COS monkey
kidney cells stimulated with a variety of ligands to
Gq-coupled receptors.

The above described kinetics with a fast and
rather complete translocation induced by BK, suggest
that PIP2 depletion after stimulation is quite
extensive. While most reports of agonist-induced PIP2
5   hydrolysis, as detected biochemically from
[^{3}H]-inositol-labeled cells, show slower and less
pronounced decreases in phosphoinositide levels,
considerable agonist- and cell type-dependent
variations exist, e.g. (Tilly et al., Biochem. J.
10   252:857-863(1988); van der Bend et al., Biochem. J 285
(Pt I):235-240 (1992); Zhang et al., Mol. Pharmacol.
50:864-869 (1996)). Where early time points were also
studied, rapid decreases in PIP2 levels have been
detected (Wijelath et al., Biochem. Biophys. Res.
15   Commun. 152:392-397 (1988); Divecha et al., EMBO J.
10:3207-3214 (1991); Stephens et al., Biochem. J. 296
(Pt 2):481-488 (1993)). For example, significant
bradykinin-induced PIP2 decreases were reported to
occur within 10 seconds in bovine aortic endothelial
20   cells (Myers et al., Cell Signal. 1:335-343 (1989)),
and at 1 minute in bombesin-stimulated 3T3 cells
(Divecha et al., supra (1991).). Rapid recovery towards
basal levels has also been found. Wijelath et al.
reported as much as 85% hydrolysis of PIP2 at 5 seconds
25   after stimulation of macrophages with interleukin,
while PIP2 levels had recovered to 50% at 60 seconds
(Wijelath et al., supra (1988)). Similar fast recovery
was also seen in other cell types (Divecha et al.,
supra (1991); Stephens et al., supra (1993)). Since
30   biochemical analyses have to rely upon measurements on
cell populations, where not all cells give synchronized
and identical responses (and many cells may not respond
at all), it is not surprising to find differences
between the results of measurements with these two
35   alternative approaches.

Characterization of fluorescence signals

        During agonist-induced translocation, several
factors may affect the fluorescent properties of these
PH domain chimeras as well as the transfer of
5   fluorescent energy between them (Tsien, Annu. Rev.
Biochem. 61:509-544 (1998)). For example, the move
away from a compartment adjacent to the lipophilic·
membrane could alter fluorescent characteristics, and
is also likely to alter FRET by increasing the degree
10   of rotational freedom. While the relative influence of
increased rotational freedom on the
translocation-induced decrease in FRET is difficult to
assess in this model sytem, fluorescence changes were
analyzed in some further detail.

15   Cells were transfected with only one of the
PLCδ1PH-CFP or PLCδ1PH-YFP constructs. After
stimulation, a small but consistent transient
fluorescence decrease was observed with either the CFP
or the YFP-tagged PH domains (Fig. 3). The original
20   green construct (PLCδ1PH-GFP) displayed similar
behavior (not shown). This transient decrease is
likely caused by fluoriphore displacement from the
membrane, since it is not observed in cells that
express a more stably membrane-anchored GFP-CAAX, nor
25   is it seen in cells that express a mutated PLCδ1PH-GFP
(R40L) (Varnai et al., J. Cell Biol. 143:501-510
(1998)) that can not bind PI(4,5)P2 and, therefore, is
cytosolic throughout the experiment. The precise
mechanism that causes this decrease of emission upon
30   cytosolic translocation is unknown; however, influence
of the local microenvironment (e.g. hydrophobicity,
charged groups, changing ion concentrations etc.) on
the spectral properties of GFP seems likely (Tsien,
supra (1998)). The "displacement" effect may explain

why the translocation-induced decrease in YFP signal
usually is somewhat larger than the increase in CFP
fluorescence. However, expressing FRET as an emission
ratio largely eliminates this effect.

5   FRET could also be measured in cells that
coexpress PLCδ1PH-CFP with YFP-CAAX (not shown);
however, using this pair, ratioing did not cancel the
above mentioned displacement effect.

          To assess the effects of construct
10 concentrations on FRET, cells expressing various levels
of the chimeric proteins were compared. Intracellular
fluorescent protein concentrations were estimated by
comparing the emission intensities of individual cells
to those of a solution of bacterially expressed,
15 purified protein of known concentration (Miyawaki et
al., supra (in press); see Experimental Procedures).
Based on these estimates, resonance could be observed
in cells with expression levels between about 2-200 µM,
over a 100-fold concentration range. However, FRET was
20 not observed in cells expressing less than about 1 µM
of each of the constructs. Very high expression
levels, on the other hand, appeared to be detrimental
to the cells (as judged from the appearance of membrane
blebs and detachment of cells 2-3 days after
25 transfection). Such cells were excluded from analysis.
These data also revealed that PLCδ1PH-CFP expression
levels (detected in fully translocated cells) did not
differ more than about 2-fold from those of PLCδ1PH-YFP
in most cells.

30   It was of interest to determine whether
estimates of CFP and YFP concentrations can be used to
calculate lipid concentrations and molecular proximity
in the cells studied. Assuming a typical attached

N1E-115 cell to be a pyramid having a 20x20 µm base and
10 µm height (having 1.3 pl volume and 1100 µm²
surface), and assuming that (I) the concentration of
both chimera is 20 µM; (II) 50% of fluoriphores are
5   located at the membrane (complete translocation roughly
doubles the fluorescence in the cytosol); (III) the
distribution of fluoriphores is homogenous along the
membrane; and (IV) fluoriphores are insensitive to the
local environment, then the calculated mean distance
10 between fluoriphores is 7-8 nm, which is close to the
reported Forster radius (50 Angstrom) for FRET between
this pair of fluoriphores (Tsien, supra (1998)).
However, it should be emphasized that these assumptions
are valid only as first approximations. For example,
15 we and others (Tall et al., Curr. Biol. 10:743-746
(2000)) noted that GFP-PH is not homogeneously
localized along the plasma membrane. Also, as
discussed above, the spectral properties of the
fluorescent proteins are sensitive to the
20 microenvironment. Nevertheless, these data set a lower
limit for the density of PIP2 molecules available for
PH binding at the inner surface of the plasma membrane.

GFP-PH rapidly shuttles between membrane and cytosol

Another important characteristic to address
25 was membrane association and dissociation rates of the
PH chimera. These rates directly influence reliability
of FRET in reporting rapid changes in PLC activity, and
are also relevant to the ability of PLC to hydrolyze
PI(4,5)P2 in cells that express high levels of the
30 PLCδ1PH-GFP protein. Accordingly, fluorescence
recovery after photobleaching (FRAP) experiments were
performed to estimate the binding and dissociation
kinetics of PLCSIPH-GFP in the membrane. Fig. 4 shows
representative results from such FRAP experiments in

N1E-115 cells. In panels A and B, the recovery rates
are depicted for GFP-CAAX and PLCδ1PH(R40L)-GFP,
constructs that are delimited to the plasma membrane
and the cytosol, respectively. The former presents the

5 extreme of slow, purely membrane-delimited diffusion
(2.81 ± 0.31 s, n=15), and the latter of fast cytosolic
diffusion (0.201 ± 0.022 s, n=15). Since FRAP of
membrane-localized PLCδ1PH-GFP is significantly faster
than that of the membrane-delimited GFP-CAAX (1.22 ±

10 0.23 s, n=40; p<0.0005; compare panel A and C), its
recovery has to be partially through the cytoplasm.
Thus, PI(4,5)P2-PH binding is a dynamic process, with
on-off rates in the order of seconds. In support of
this notion, FRAP times further decreased during

15 agonist-induced partial translocation, when association
rates are increased due to the raised cytosolic GFP-PH
levels (panel D). The rapid shuttling between membrane
and cytosol of individual PLCδ1PH-GFP molecules could
explain why PI(4,5)P2 is still available for

20 PLC-mediated hydrolysis or for binding of other
proteins in cells expressing these chimeras.

Widefield FRET detection allows prolonged monitoring
independent of cell shape changes.

Rapid confocal scanning of cells transfected

25 with PLCδ1PH-GFP leads to considerable photobleaching
(within 100 frames) and often causes severe phototoxic
damage, manifested as membrane blebbing and loss of
membrane integrity within minutes. Using wide-field
optical detection and integrating emission from an

30 entire cell (or even clusters of cells) allowed
excitation intensity to be dimmed by as much as 100 to
>1000 fold, while still retaining acceptable
signal-to-noise ratio. Thus, FRET can be followed in
single cells for extended periods of time without

detectable cell damage. This permits recording of
complex stimulation protocols, as shown in Fig. 5A. As
shown therein, a single N1E-115 cell that is repeatedly
stimulated with short pulses of neurokinin A (NKA) from

5 a puffer pipette showed repeated PLC activation. The
response to NKA displays incremental partial homologous
desensitization of PLC activation, while the response
to subsequently added BK is unaltered. Optimizing for
low excitation intensity, recordings of several hours

10 can be obtained with sub-second resolution.

In N1E-115 and other cells, addition of
certain agonists causes rapid and significant shape
changes. For instance, LPA causes neurites to retract
and the cell soma to round up within 60 seconds (Jalink

15 et al., Cell Growth Differ. 4:247-255 (1993)). In
contrast, addition of BK has opposite effects,
promoting a differentiated phenotype (van Leeuwen et
al., Nat. Cell Biol. 1:242-248 (1999)). During
confocal imaging, such shape changes (as well as the

20 slight drift in focal plane that inevitably occurs over
prolonged times) seriously complicate the
quantification of GFP-PH translocation. Since FRET
analysis uses the total integrated emission from a
cell, shape changes and focal drift do not present

25 problems.

In very flat and small cell structures such
as neurites and lamellipodia (below approximately 2 μm
in thickness), confocal imaging cannot detect
translocation due to its inherent limit in z-axis

30 resolution. However, in such cases changes in FRET can
still be reliably detected as shown by the
agonist-induced PLC activation recorded over a single
neurite (Fig. 5B). FRET can also be recorded from cell
populations (Fig. 5C) providing with an average

response that would need analysis of hundreds of single
cell recordings. Thus, detecting resonance between
fluorescent protein-labeled PH domains overcomes a
number of the limitations that are associated with
5   confocal detection.

Determination of whether FRET reports changes in
membrane PI(4,5)P2 or increases in cytosolic IP3

        While PLCδ1PH-GFP has been introduced as an
indicator of membrane PI(4,5)P2 (Stauffer et al., Curr.
10   Biol. 8:343-346 (1998); Varnai et al., supra (1998)),
it also displays high affinity to IP3 (Hirose et al.,
Science 284:1527-1530 (1999)) which may exceed its
affinity to PI(4,5)P2, although it is difficult to
accurately measure the latter as it is displayed in
15   vivo. Based on such relative affinity estimates,
Hirose and coworkers recently suggested that
PLCδ1PH-GFP actually monitors IP3 increases rather than
the changes in lipid levels in MDCK cells (Hirose et
al., supra (1999)). They reported that microinjection
20   of IP3 in MDCK cells was sufficient to cause
displacement of PLCδ1PH-GFP from the membrane to the
cytosol through competition for binding of the
fluorescent construct to membrane PI(4,5)P2. They also
showed that expression of an IP3-5-phosphatase
25   completely blocked the agonist-induced translocation of
the fluorescent protein, and concluded that PI(4,5)P2
changes do not make a significant contribution to the
translocation response during stimulation.

        While FRET analysis effectively monitors the
30   result of PLC activation regardless of whether it is
the lipid decrease or the IP3 increase that is more
important for the translocation response, this question
deserved a more detailed analysis. First it was

determined whether intracellular applications of IP3
that generate a Ca^{2+} signal comparable to that evoked
by an agonist would cause translocation of the PLCδ1PH
that is similar to what is caused by agonist

5   stimulation. N1E-115 cells were loaded with 20 pM of
the calcium indicator Fura red and 100 pM caged IP3 by
in situ high frequency electroporation. Unlike
microinjection, this technique allows setting of the
final concentration of caged IP3 in the cytosol with

10   high precision (see Experimental Procedures), as
confirmed by the observation that upon electroporation,
intracellular and extracellular fluorescence levels
were equal. As shown in Fig. 6A, UV flash photolysis
of 1 PM of caged IP3 rapidly mobilized Ca^{2+} from

15   internal stores, with no visible translocation of
PLCδ1PH-GFP to the cytosol. Subsequent release of 10
μM of caged IP3 caused a higher Ca^{2+} response and a
small translocation. Only high IP3 concentrations that
evoked a large and prolonged Ca^{2+} increase were able to

20   displace PLCδ1PH-GFP from the plasma membrane. In
contrast, BK stimulation caused a larger translocation
response than the highest amounts of IP3 with a Ca^{2+}
signal that was comparable to that induced by the
smallest amount of IP3 (Fig. 6A). In cells

25   electroporated with no caged IP3 in the electroporation
buffer, intense UV flashes did not influence
intracellular Ca^{2+} levels, membrane localization of the
chimera, or any of the BK-induced changes herein (not
shown).

30   Next, the effects of interfering with
PI(4,5)P2 resynthesis on the kinetics of translocation
in N1E-115 cells was studied. PI(4,5)P2 resynthesis
was inhibited by low concentrations (5 μM) of phenyl
arsine oxide (PAO) (Fig. 6B) or quercetin (Wiedemann et

35   al., EMBO J. 15:2094-2101 (1996)), or by depletion of

64

free inositol using prolonged incubation in
inositol-free medium (not shown).In PAO-treated cells,
BK induced a sustained translocation of PLCδ1PH-GFP to
the cytosol, while IP3 increases in such cells are only
5   transient (Hunyady et al., J. Biol. Chem. 266:2783-2788
(1991)). In control experiments, these pretreatments
did not influence signaling events such as BK-induced
Ca^{2+} signaling (peak Ca^{2+} values of 870 ± 130 nM in
control cells, and 845 ± 114 nM, in PAO pretreated
10 cells, n=6, mean ± SEM) or the thrombin- and
lysophosphatidate-induced actinomyosin contraction
(Jalink et al., supra (1993); Jalink et al., J. Cell
Biol. 118:411-419 (1992)). Similar observations were
made in HEK293 cells (not shown), suggesting that the
15 translocation of PH domains under these conditions
reports the depleted PI(4,5)P2 pool rather than the
transient IP3 increase.

Moreover, when adrenal glomerulosa cells were
stimulated with angiotensin II in the presence of Sr^{2+},
20 a condition under which IP3 metabolism via
Ins(1,3,4,5)P4 is greatly reduced (Balla et al., supra
(1994)), hence yielding significantly higher
Ins(1,4,5)P3- and diminished Ins(1,3,4)P3 increases
(Fig.6 C,D), the translocation of PLCδ1PH-GFP was not
25 significantly different (Fig. 6 E) from that observed
in the presence of Ca^{2+}. Translocation responses of
N1E-115 cells in response to BK were also similar in
the presence of Ca^{2+} or Sr^{2+} (Fig. 6 F).

Taken together, these results indicate that,
30 at least for the cells and agonists described above,
PLCδ1PH-GFP translocation primarily reports changes in
membrane PI(4,5)P2 content and not IP3 increases. The
reason for the apparently stronger binding of PLCδ1PH
to membranes observed in live cells compared to the

reported low in vitro affinity (Hirose et al., supra
(1999)) to PIP2 containing lipid vesiclés or BiaCore
surface (Lemmon et al., Proc. Natl. Acad. Sci. U.S.A
92:10472-10476 (1995)) is unclear at present, but may
5   indicate a more complex interaction of the PLCδ1PH
domain with the native membranes that is not mimicked
by the in vitro experiments. However, the finding
reported in Hirose et al., supra (1999) that high IP3
levels can make significant contributions to the
10   translocation response was confirmed. Whether such
high levels or IP3 occur under the experimental
conditions used with intact cells remains to be
elucidated. Nevertheless, possible interference from
large IP3 increases should be kept in mind during
15   interpretations of the results of such translocation
experiments.

FRET reveals response heterogeneity to different GPCR
agonists that is not reflected in Ca^{2+} mobilization.

Having characterized the use of FRET between
20   CFP and YFP-tagged PH domains of PLCδ1 to record PLC
activation, the kinetics of responses to a set of
calcium-mobilizing GPCR agonists were compared.
Included in this panel were the peptide agonists BK and
NKA, as well as the bioactive lipid lysophosphatidate
25   (LPA), the protease thrombin, and the bioactive amine,
histamine. Thrombin and LPA, in addition to inducing
Ca^{2+} mobilizations from internal stores, are also
strong inducers of Rho-dependent remodeling of the
actin cytoskeleton in these cells (Jalink et al., supra
30   (1993); Jalink et al., supra (1992)). Histamine, on
the other hand, does not induce Rho-dependent actin
remodeling, but is known to induce Ca^{2+} oscillations in
several cell types (e.g. Paltauf-Doburzynska et al.,

J.Physiol.(Lond) 524 Pt.3:701-713 (2000); Zhu et al.,
J. Biol. Chem. 275:6063-6066 (2000))

     These agonists evoke very similar Ca^{2+}
mobilizations in N1E-115 cells, characterized by a fast
5   onset and rapid termination well within 2 minutes (Fig.
7). Estimated peak Ca^{2+} levels ranged from 0.6-2 µM,
and, again, showed no consistent differences between
agonists. When PLC activation patterns were recorded
by FRET analysis, using the same agonists under
10   identical conditions, several distinct profiles of PLC
activation kinetics were obtained (Fig. 7). First,
both NKA and BK caused fast and near-complete
translocation of the probe. This response was
transient, returning to baseline within 2-5 minutes.
15   Stimulation with thrombin or LPA evoked a different
type of response: these translocations had slower onset
and smaller amplitude, averaging 25% of BK response
control values (n=22). They also returned to baseline
at a slower rate. The response to histamine was much
20   slower and of small amplitude (40% of BK-induced peak
values, n=15), but it was long-lasting (at least for 15
minutes, but often much longer).

     Differences in degree of PIP2 hydrolysis
induced by activation of different Gq-coupled receptors
25   have also been reported (van der Bend et al., supra
(1992), Tilly et al., Biochem. J. 266:235-243 (1990))
in biochemical studies. However, so far only cytosolic
Ca^{2+} responses could be used to analyze receptor
activation patterns at the single cell level. On the
30   other hand, the shape of the Ca^{2+} response is
determined by several other factors: it can be
triggered at relatively low levels of IP3 and its shape
is also determined by the Ca^{2+}-induced Ca^{2+} release and
inactivation properties of the IP3-receptor-channels,

as well as by the activities of the various Ca^{2+} sequestration mechanisms. The present approach provides an opportunity to study a more upstream receptor-mediated event, namely PLC activation, and its
5   regulation in detail at the single cell level.

PH domain translocation kinetics mirror receptor activation.

     These results thus suggest that PLC activation as assessed by FRET is a more faithful index
10   of receptor activity than the more distal Ca^{2+} transients. However, inactivation could occur at various steps in the signal cascade, including at the levels of receptor, G protein and PLC and, conceivably, also by modulation (upregulation) of PI(4,5)P2
15   resynthesis. To test whether there is desensitization at the level of PLC, G proteins were directly activated using AlF^{4-} (Fig. 8A). While onset of AlF^{4-} induced PLC activation was slow, no desensitization was observed in any of these experiments. Similarly, cells expressing
20   a constitutively active Gαq mutant showed mostly cytosolic localization of PLCδ1 PH-GFP domains for at least 2 days (Fig. 8B). Control transfection with activated Gα12 had no effect. At lower expression levels, the activating mutant Gαq induced sustained
25   partial translocation that also persisted for several days. These experiments suggested that no significant desensitization occurs downstream of Gq and PLC. In line with this notion, significant heterologous desensitization between sequentially added agonists was
30   not observed (compare e.g. Fig. 5 and 7, last panel), whereas prolonged exposure of cells to each individual agonist induced complete (homologous) desensitization.

To further determine whether such monitoring
of PLC activity truly follows receptor activity (in
other words coupling and uncoupling between receptors
and G proteins), the FRET responses of N1E-115 cells

5   expressing either the wild-type NK2 receptors or a
C-terminally truncated form, which is greatly impaired
in its ability to desensitize (Alblas et al., J. Biol.
Chem. 270: 8944-8951 (1995)), were compared. After
stimulation of the wild-type human NK2 receptors the

10   translocation response decays towards baseline within
minutes (average 50%) recovery time 83 ± 38 s, n=25;
compare Fig. 7 and 8C). Application of short pulses of
agonist using a puffer pipette resulted in incomplete
desensitization, and decayed significantly faster (45 ±

15   7 s, n=60, Fig. 8C) between applications of stimuli due
to the rapid dissociation of the ligand from the
receptor (Vollmer et al., J. Biol. Chem.
274:37915-37922 (1999)). Conversely, stimulation of a
C-terminally truncated mutant human NK2 receptor, that

20   was reported to be transforming in Rat-1 fibroblasts,
and which has been found to display prolonged coupling
to PLC (Alblas et al., supra (1995); Alblas et al.,
EMBO J. 15:3351-3360 (1996); Alblas et al., J. Biol.
Chem. 268:22235-22238 (1993)) induced a much prolonged

25   cytosolic translocation as assessed in FRET analysis
(Fig. 8C). However, in the majority of cells, the FRET
signal eventually slowly returned to baseline (Fig. 8D;
note the different time scale), with an average 50%
recovery time of 1365 ± 599 s (n=19) in the truncated

30   receptor. This result indicates the existence of an
alternative and much slower desensitization mechanism
that functions even in NK2 receptors lacking the
C-terminus. The kinetics of this slow desensitization
closely paralleled those of receptor internalization

35   (not shown), suggesting that one of the main
determinants for termination of NKA-induced PLC

signaling could be receptor internalization. Analysis of receptor activity by monitoring PLC activity by FRET will greatly aid further studies addressing these questions in more detail.

5   In summary, described above is a fluorescence resonance-based detection scheme of membrane localization of tagged PLCδ1 PH domains for analysis of activation-inactivation kinetics of PLC in single cells with high temporal resolution. This method has a number
10   of significant advantages over confocal detection of membrane localization, including: (i) a significant decrease in excitation intensity allowing prolonged experiments or very fast sampling with little photobleaching and phototoxicity; (ii), suitability for
15   very flat cells such as fibroblasts and motile cells; (iii), extendibility to record from cell populations as well as from small subregions such as neurites; and (iv) a simpler detection hardware. FRET detection of PLC activation is a fairly robust response that can be
20   routinely obtained in a variety of cell types.

Analysis of the translocation responses suggests that localization of PLCδ1PH-GFP largely reports PI(4,5)P2 dynamics, although at high concentrations IP3 can also contribute to translocation
25   of the PH domains to the cytosol. Comparison of the Ca^{2+} and FRET-recorded responses of several agonists of GPCRs suggest that PLC activation detected by FRET is a more faithful reflection of receptor activity than the Ca^{2+} signal and that little if any "desensitization" or
30   "uncoupling" occurs beyond the levels of G proteins.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

5   Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

71

What is claimed is:

    1.   A membrane molecule indicator, said indicator
comprising:
5   (a) at least one membrane molecule indicator
domain;
         (b) a donor fluorescent domain; and
         (c) an acceptor fluorescent domain;
wherein fluorescence resonance energy transfer (FRET)
10 between said donor domain and said acceptor domain is
indicative of a property of said membrane molecule.

    2.   The indicator of claim 1, which comprises one
polypeptide.

    3.   The indicator of claim 1, which comprises two
15 polypeptides.

    4.   The indicator of claim 1, further comprising a
membrane anchoring domain.

    5.   The indicator of claim 1, further comprising a
linker between said donor fluorescent domain and said
20 acceptor fluorescent domain.

    6.   The indicator of claim 1, comprising two
membrane molecule indicator domains.

    7.   The indicator of claim 6, wherein each of said
two membrane molecule indicator domains associates with
25 the same type of membrane molecule.

72

8.   The indicator of claim 1, wherein the property indicated by said membrane molecule indicator is selected from the group consisting of localization, abundance, conformation and post-translational

5   modification state of said membrane molecule.

9.   The indicator of claim 1, wherein said membrane molecule is a lipid.

10.   The indicator of claim 9, wherein said lipid is phosphatidylinositol 4,5-bisphosphate (PIP2).

10   11.   The indicator of claim 10, wherein said membrane molecule indicator domain is a pleckstrin homology (PH) domain.

12.   The indicator of claim 11, wherein said PH domain is a PLCδ1 or PLCβ PH domain.

15   13.   The indicator of claim 1, wherein said donor fluorescent domain is selected from the group consisting of a GFP and a dsRED.

14.   The indicator of claim 1, wherein said donor fluorescent domain is a CFP.

20   15.   The indicator of claim 1, wherein said acceptor fluorescent domain is selected from the group consisting of a GFP and a dsRED.

16.   The indicator of claim 1, wherein said acceptor fluorescent domain is a YFP.

25   17.   The indicator of claim 4, wherein said membrane anchoring domain comprises a CaaX motif.

18. A cell comprising the indicator of any of claims 1-17.

19. A nucleic acid molecule which encodes a membrane molecule indicator, or a nucleic acid kit, the
5 nucleic acid molecule components of which encode a membrane molecule indicator, said indicator comprising:
       (a) at least one membrane molecule indicator domain;
       (b) a donor fluorescent domain; and
10   (c) an acceptor fluorescent domain;
wherein fluorescence resonance energy transfer (FRET) between the donor domain and the acceptor domain is indicative of a property of said membrane molecule.

20. The nucleic acid molecule or kit of claim 19,
15 wherein the encoding nucleotide sequences are operatively linked to one or more promoters of gene expression.

21. The nucleic acid molecule or kit of claim 19, wherein said indicator comprises one polypeptide.

20   22. The nucleic acid molecule or kit of claim 19, wherein said indicator comprises two polypeptides.

23. The nucleic acid molecule or kit of claim 19, wherein said indicator further comprises a membrane anchoring domain.

25   24. The nucleic acid molecule or kit of claim 19, wherein said indicator further comprises a linker between said donor fluorescent domain and said acceptor fluorescent domain.

25. The nucleic acid molecule or kit of claim 19, wherein said indicator comprises two membrane molecule indicator domains.

26. The nucleic acid molecule or kit of claim 25,
5 wherein each of said two membrane molecule indicator domains associate with the same type of membrane molecule.

27. The nucleic acid molecule or kit of claim 19, wherein the property indicated by said membrane
10 molecule indicator is selected from the group consisting of localization, abundance, conformation and post-translational modification state of said membrane molecule.

28. The nucleic acid molecule or kit of claim 19,
15 wherein said membrane molecule is a lipid.

29. The nucleic acid molecule or kit of claim 28, wherein said lipid is PIP2.

30. The nucleic acid molecule or kit of claim 29, wherein said membrane molecule indicator domain is a PH
20 domain.

31. The nucleic acid molecule or kit of claim 30, wherein said PH domain is a PLCδ1 or PLCβ PH domain.

32. The nucleic acid molecule or kit of claim 19, wherein said donor fluorescent domain is selected from
25 the group consisting of a GFP and a dsRED.

33. The nucleic acid molecule or kit of claim 19, wherein said donor fluorescent domain is a CFP.

34. The nucleic acid molecule or kit of claim 19, wherein said acceptor fluorescent domain is selected from the group consisting of a GFP and a dsRED.

35. The nucleic acid molecule or kit of claim 19,
5 wherein said acceptor fluorescent domain is a YFP.

36. The nucleic acid molecule or kit of claim 23, wherein said membrane anchoring domain comprises a CaaX motif.

37. A cell expressing the nucleic acid molecule
10 or nucleic acid molecule kit components of any of claims 19-36.

38. A method of determining a property of a membrane molecule in a cell, comprising:
   (a) providing a cell comprising the membrane
15 molecule indicator of any of claims 1-17; and
   (b) determining FRET between said donor fluorescent domain and said acceptor fluorescent domain,
   wherein FRET between said donor domain and said
20 acceptor domain is indicative of a property of said membrane molecule.

39. The method of claim 38, wherein said cell recombinantly expresses a known or candidate signaling molecule.

25 40. The method of claim 39, wherein said signaling molecule is a G-protein coupled receptor.

41. A method of identifying a compound that modulates a property of a membrane molecule, comprising:

(a) contacting a cell comprising the membrane

5 molecule indicator of any of claims 1-17 with one or more test compounds, wherein said cell further comprises said membrane molecule; and

(b) determining FRET between said donor fluorescent domain and said acceptor fluorescent domain

10 following said contacting,

wherein increased or decreased FRET following said contacting indicates that said test compound is a compound that modulates a property of said membrane molecule.

15   42. The method of claim 41, wherein said contacting is by administration of said test compound to the exterior of said cell.

43. The method of claim 41, wherein said contacting is by recombinant expression of said test

20 compound in said cell.

44. The method of claim 41, wherein said compound that modulates a property of said membrane molecule is selected from the group consisting of a receptor agonist, antagonist, and inverse agonist.

25   45. The method of claim 41, wherein said cell recombinantly expresses a known or candidate signaling molecule.

46. The method of claim 45, wherein said known or

30 candidate signaling molecule is a G-protein coupled receptor.
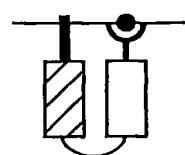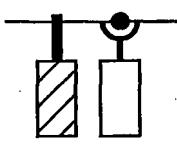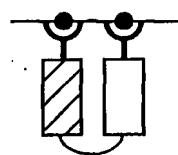
Fig. 1A   Fig. 1B   Fig. 1C   Fig. 1D
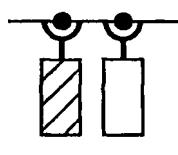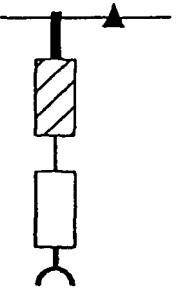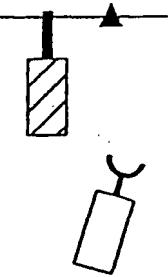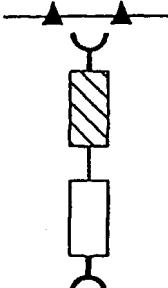
Fig. 1E   Fig. 1F   Fig. 1G   Fig. 1H
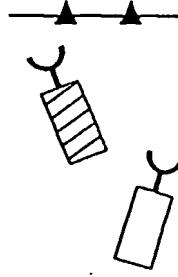
Fig. 1I   Fig. 1J   Fig. 1K   Fig. 1L

Fig.2A

Fig.2B

Fig.2C
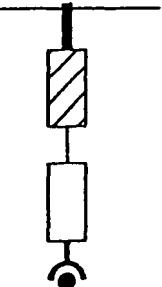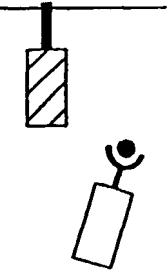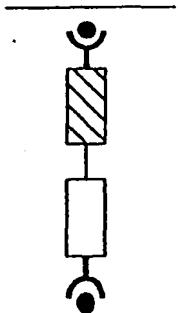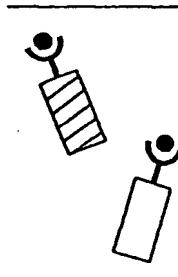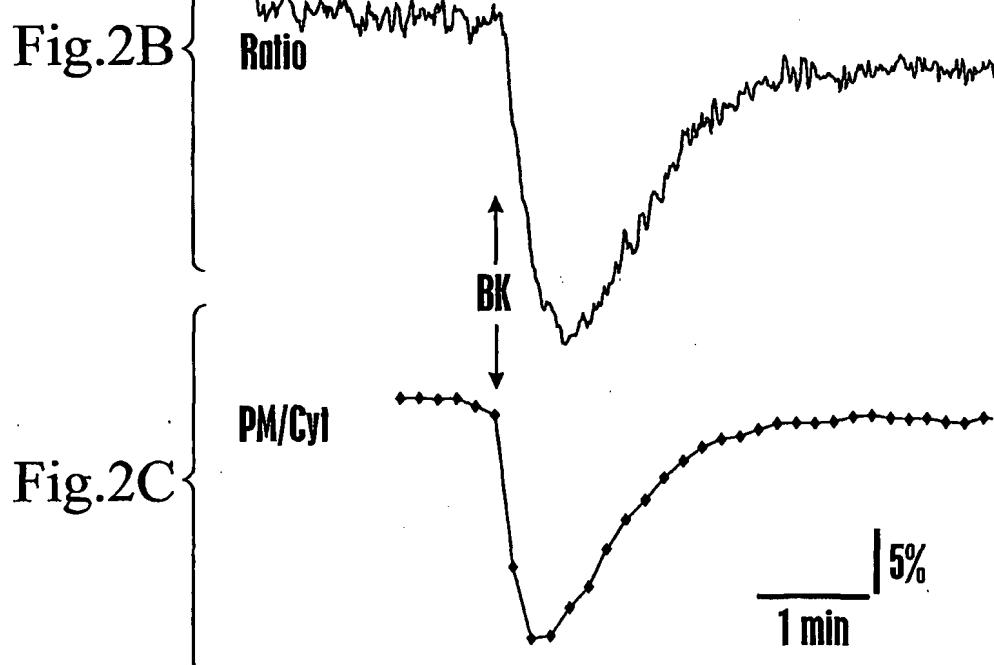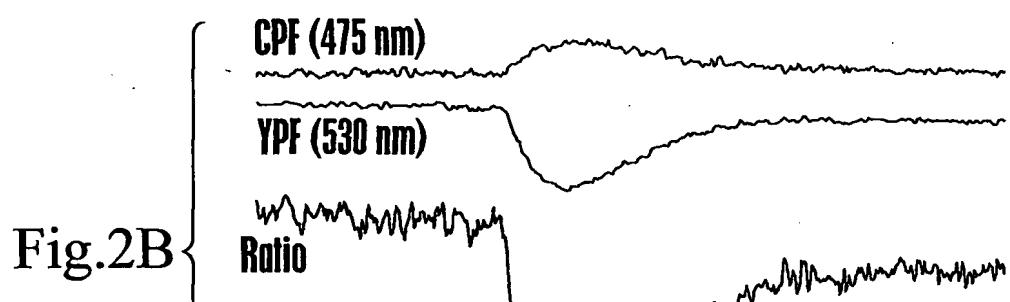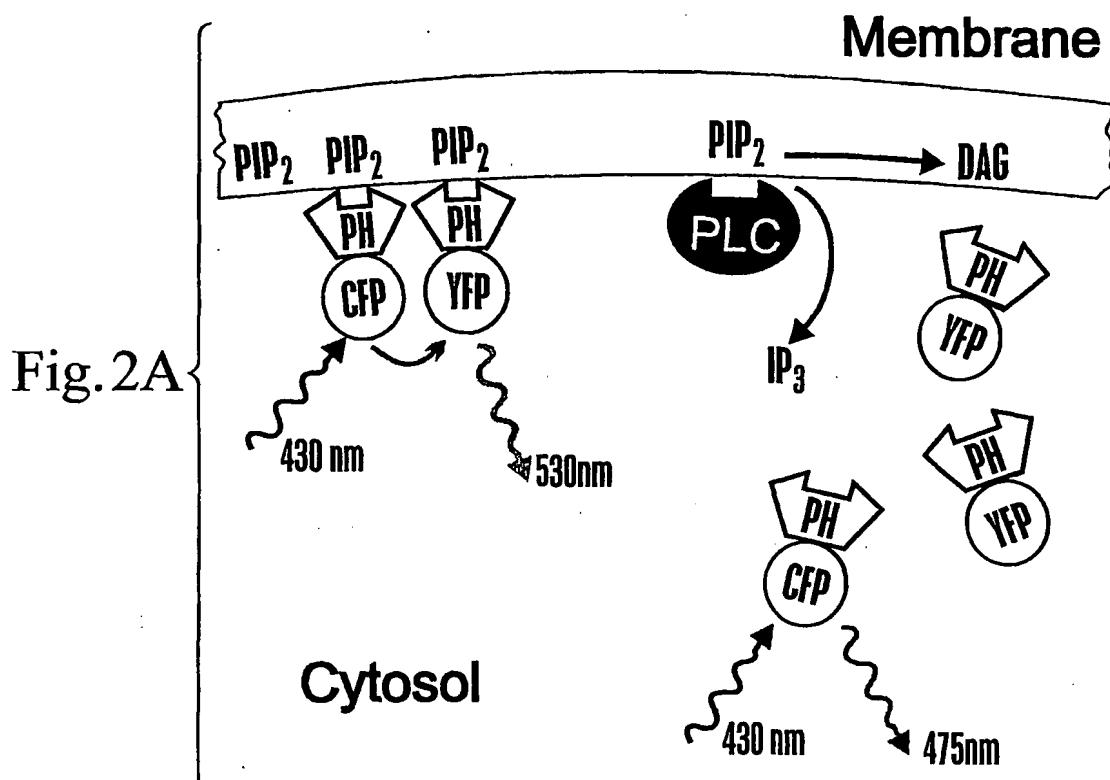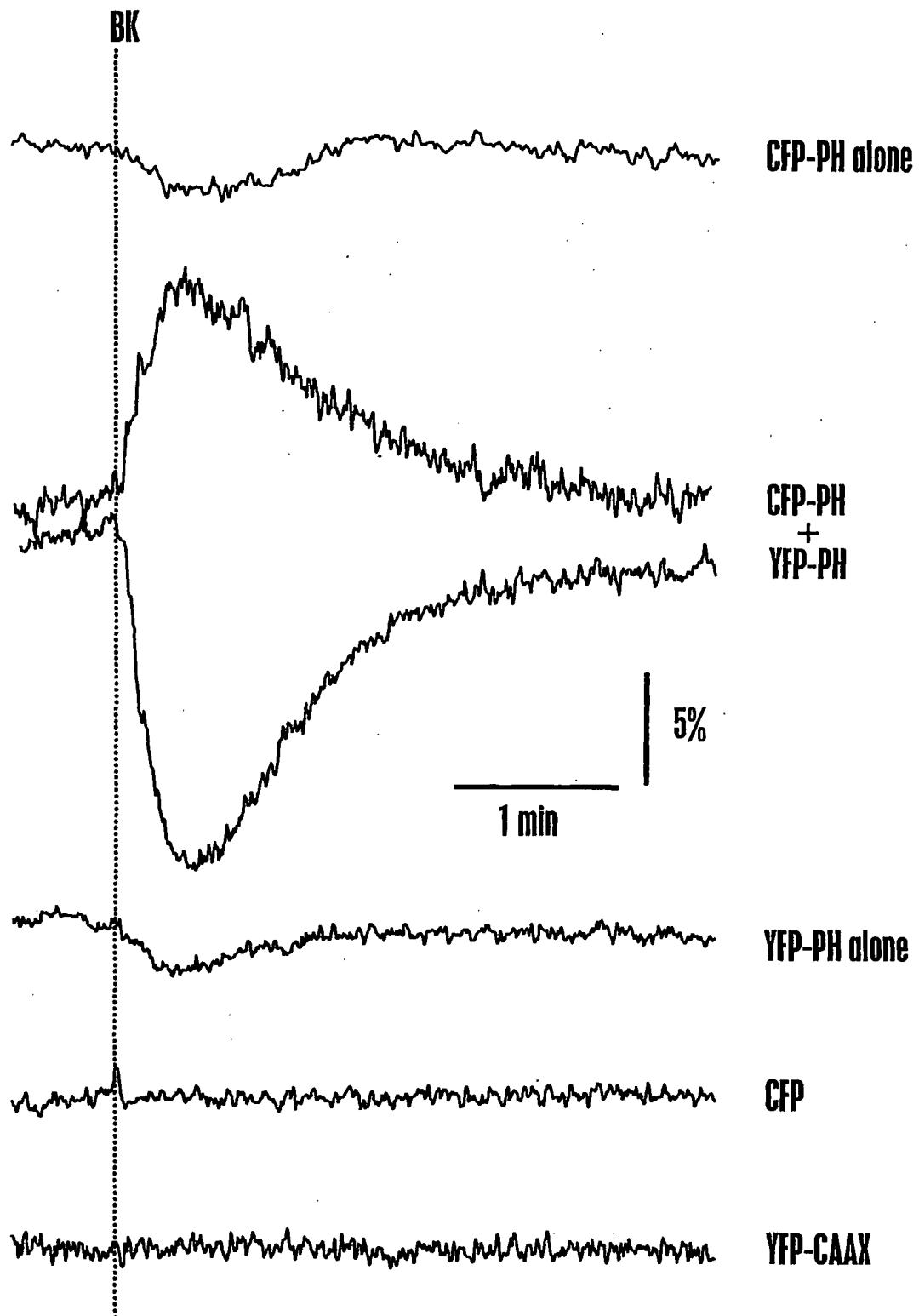
Fig. 3

Fig. 4A

YFP-CAAX
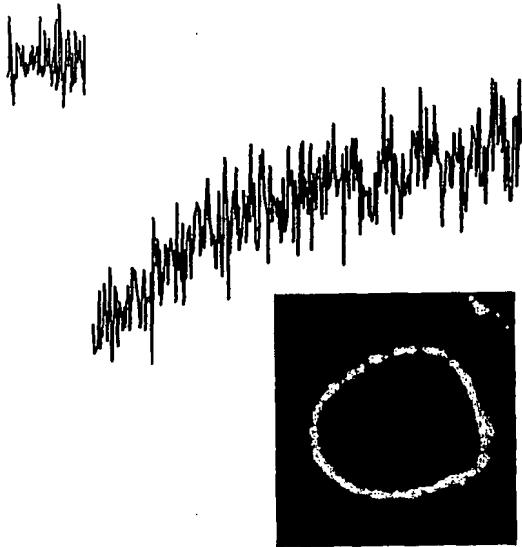
Fig. 4B

GFP-PHmut
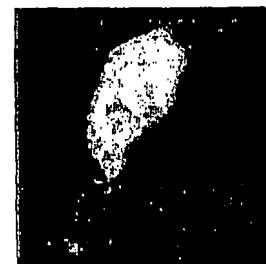
Fig. 4C

GFP-PH
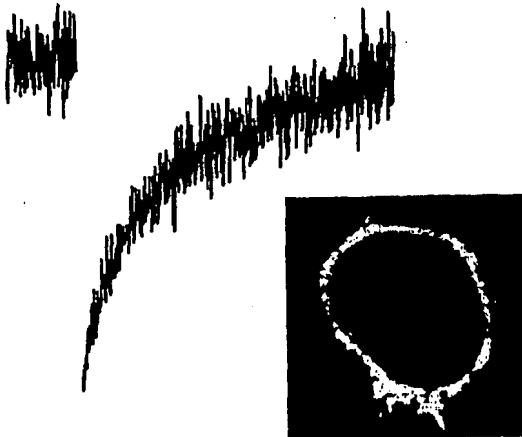
# Fig. 4D

GFP-PH (translocated)
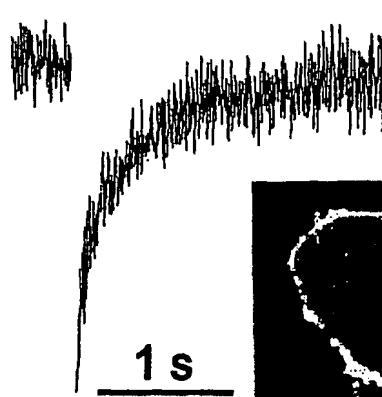
1 s
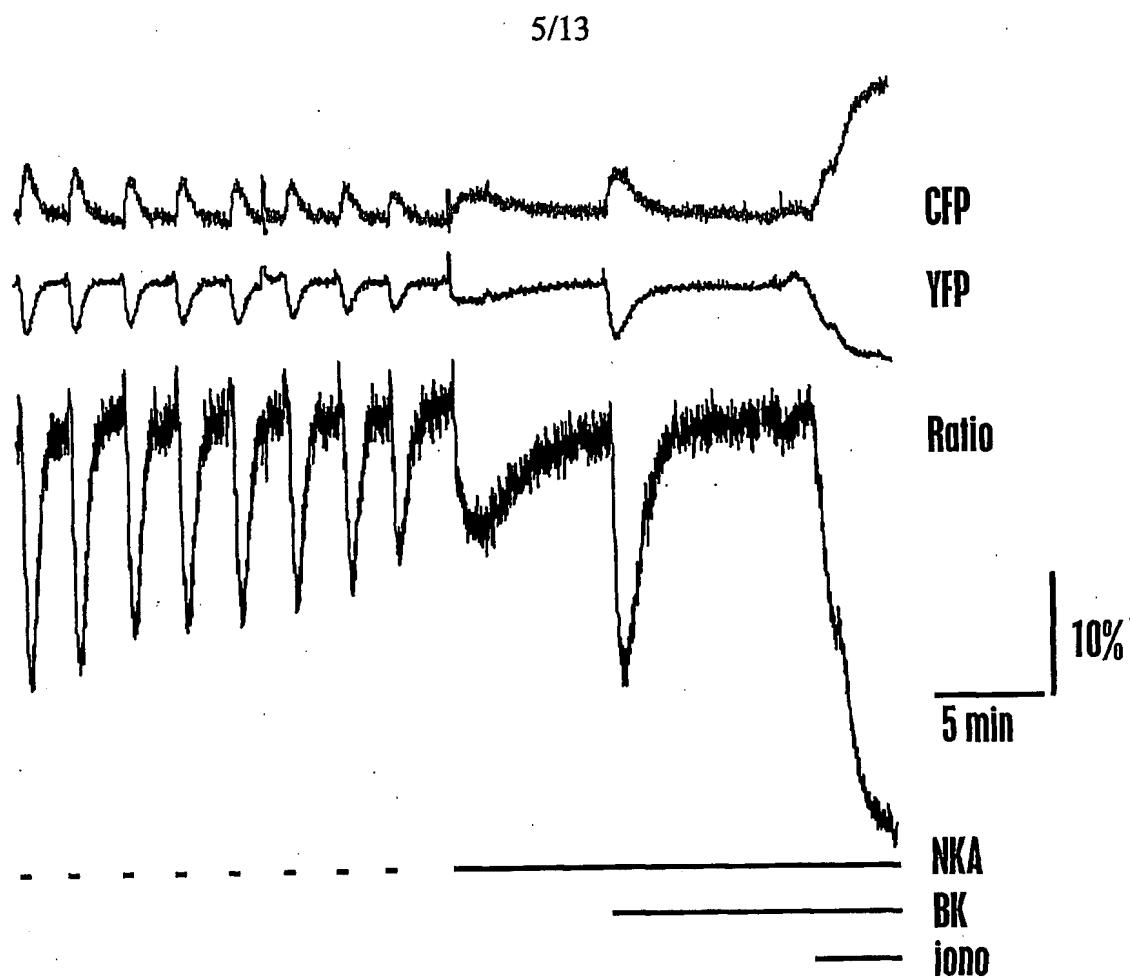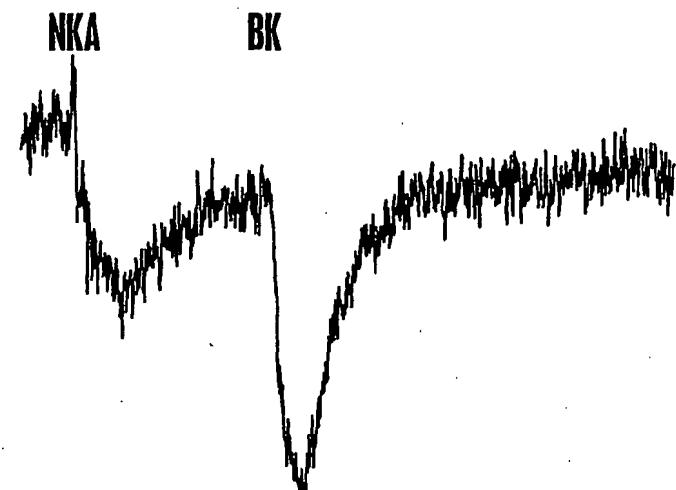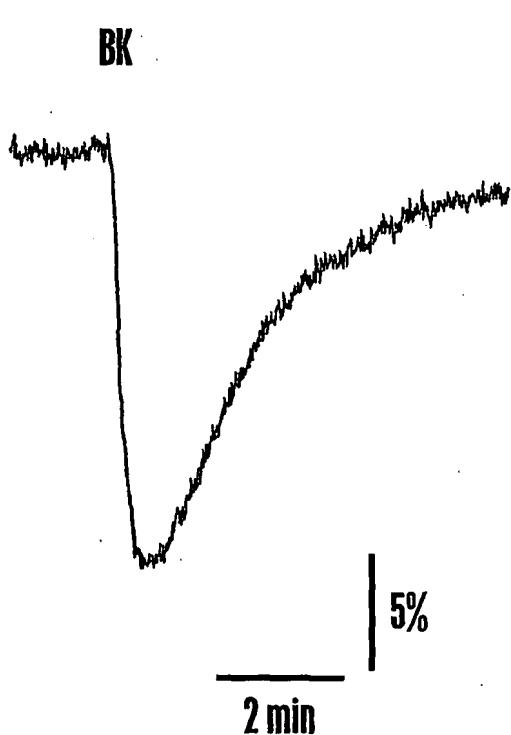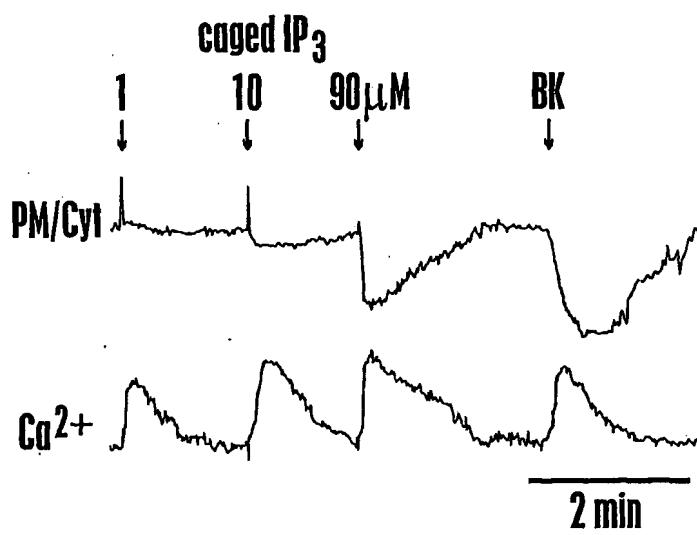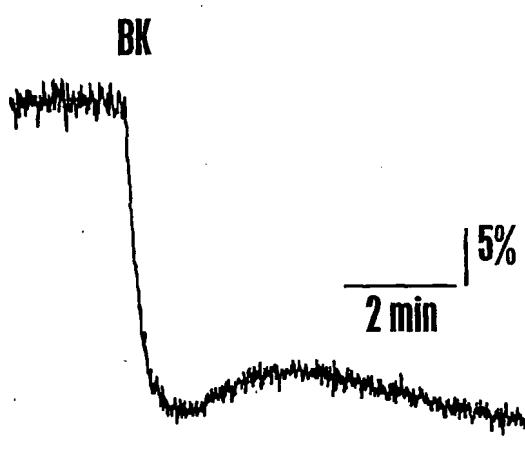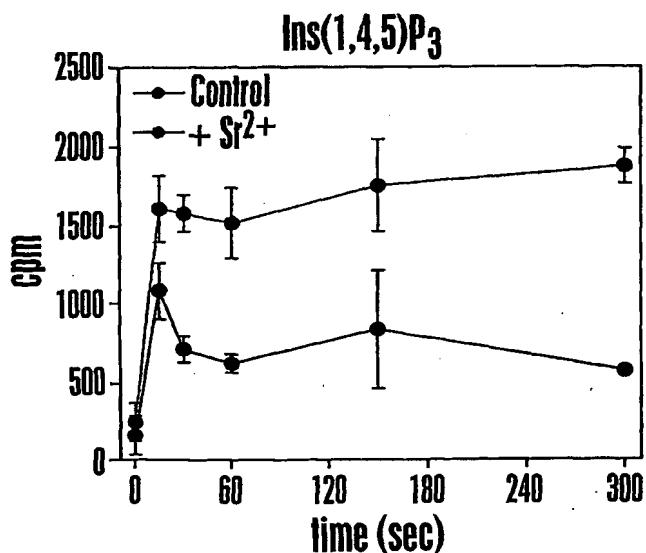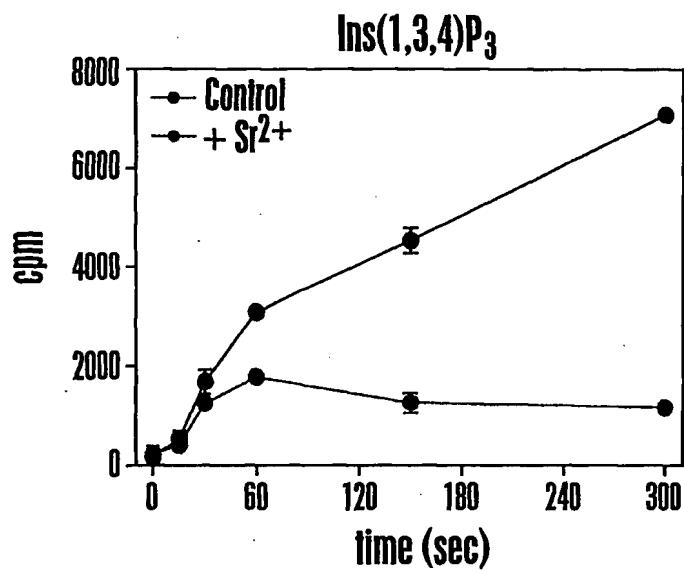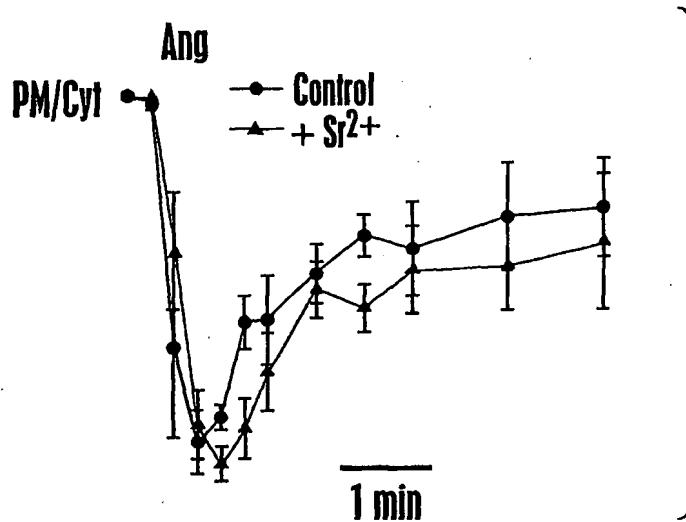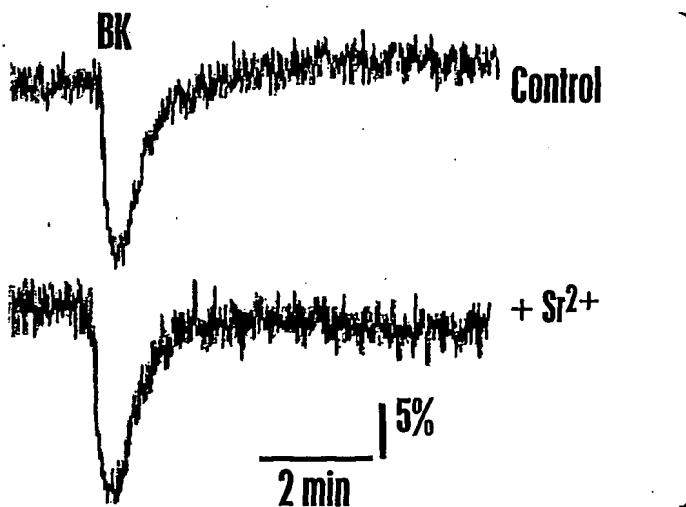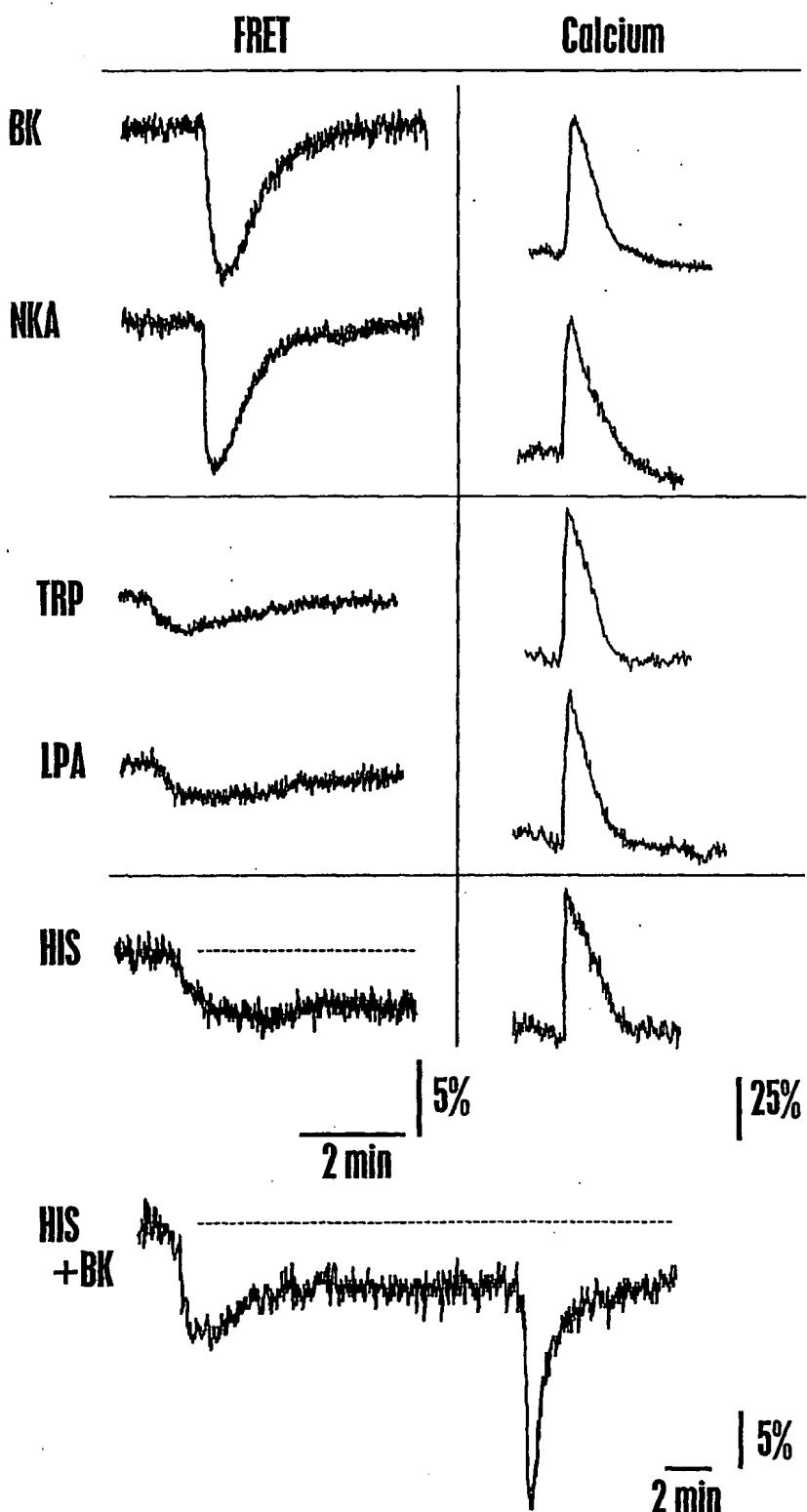
5/13

Fig. 5A

Fig. 5B

Fig. 5C

Fig. 6A

Fig. 6B

Fig. 6C

Fig. 6D

Fig. 6E

Fig. 6F

Fig. 7

Fig. 8A

Fig. 8B

Fig. 8C

Fig. 8D
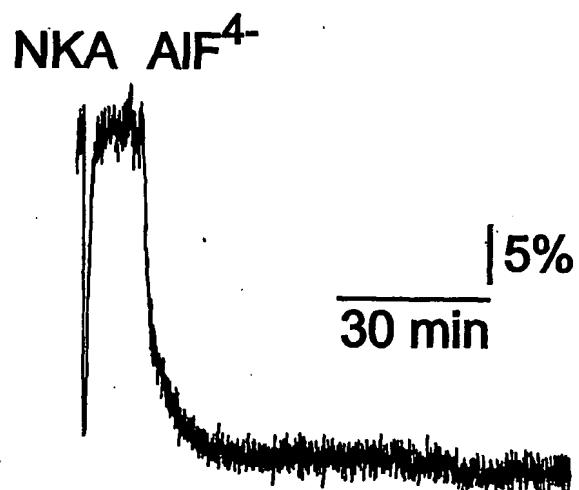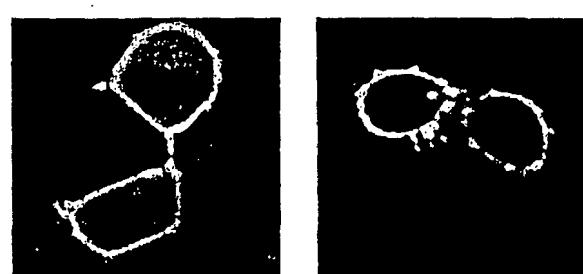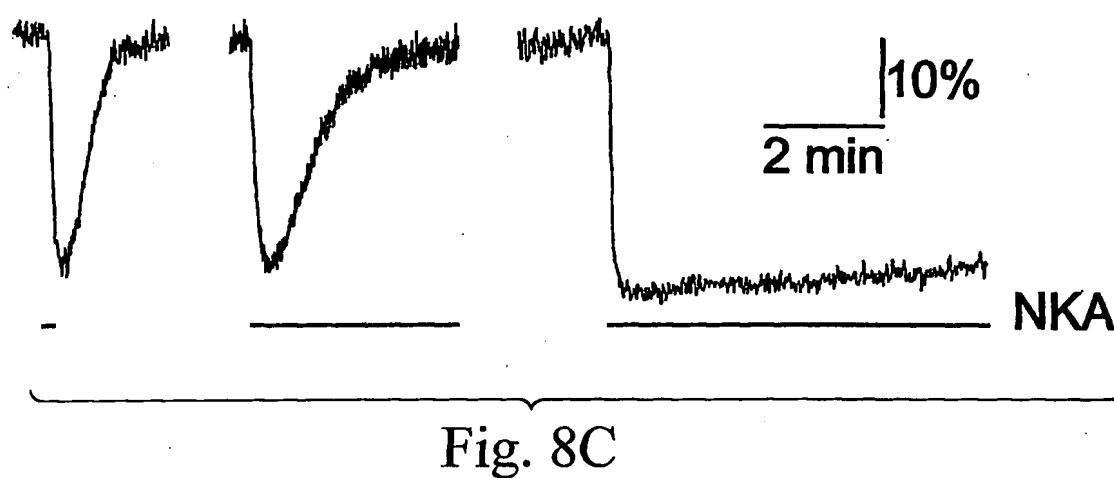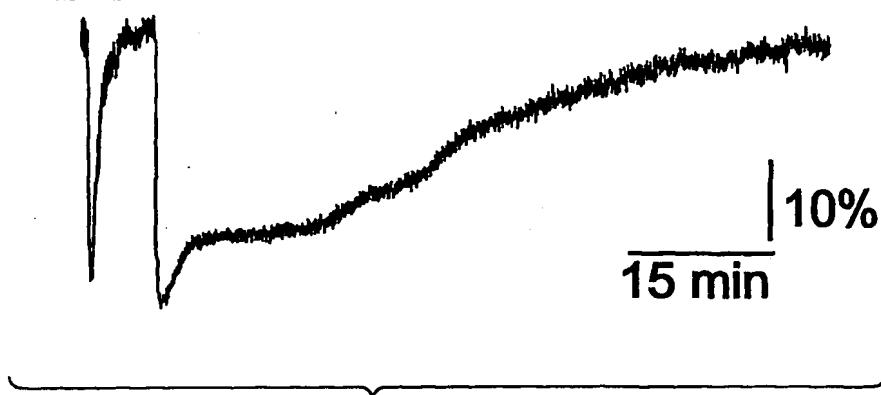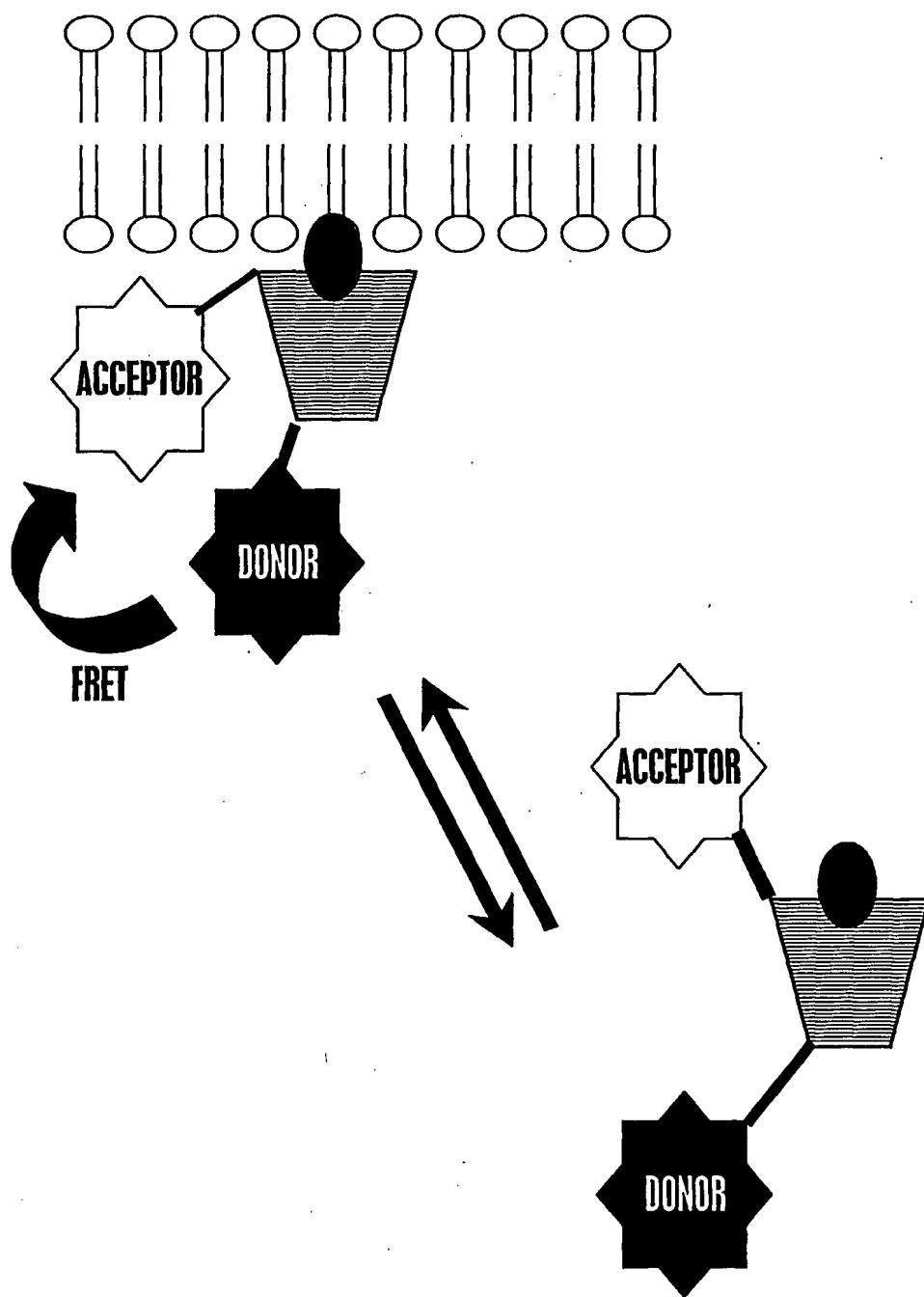
Fig. 9
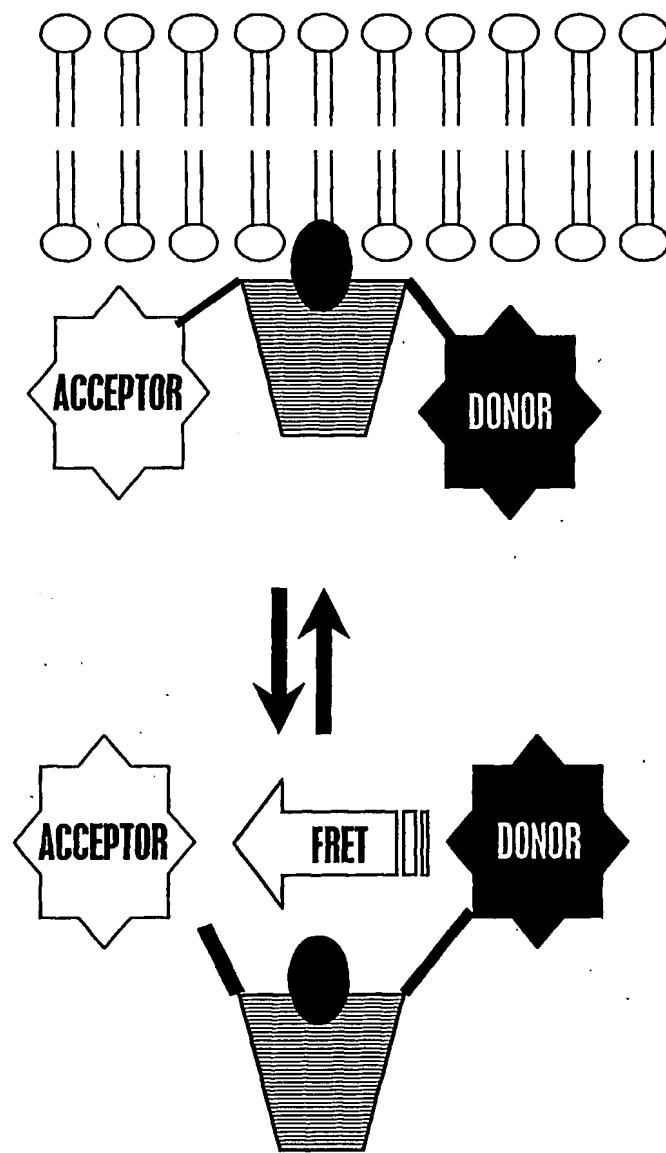
Fig. 10

SEQUENCE LISTING

<110> The Netherlands Cancer Institute
      Jalink, Kees

<120> Membrane Molecule Indicator Compositions
   and Methods

<130> P 58962

<150> US 60/250,679
<151> 2000-11-30

<150> US 60/256,559
<151> 2000-12-18

<160> 8

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<221> VARIANT
<222> 2
<223> Xaa=R or K

<221> VARIANT
<222> 7
<223> Xaa=any amino acid

<400> 1
Arg Xaa His His Cys Arg Xaa Cys Gly
 1   5

<210> 2
<211> 4
<212> PRT
<213> Homo sapiens

<400> 2
Lys Asp Glu Leu
 1

```
<210> 3
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 3
cctgcggccg cggtaccgat atcagatgtt gagctccttc ac                    42



<210> 4
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 4
ccgaattccc gggtctcagc catggactcg ggccgggact tc                    42



<210> 5
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 5
ggctgagacc cgggaattcg gcttgtacag ctcgtccatg                       40



<210> 6
<211> 20
<212> PRT
<213> Homo sapiens

<400> 6
Lys Met Ser Lys Asp Gly Lys Lys Lys Lys Lys Ser Lys Thr Lys
 1               5                  10                  15
Cys Val Ile Met
            20
```

```
<210> 7
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 7
ccgaattccc gggtcaagat gagcaaagat ggtaaaaag                    39


<210> 8
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 8
cctgcggccg cggtaccgag atctttacat aattacacac tt               42
```